JOURNAL

OF

BACTERIOLOGY

VOLUME 3[illegible]

BALTIMORE, MD.
1937

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VIRUSES AND KOCH'S POSTULATES[1]

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Diseases at one time were thought to be caused by wrath of the gods, configuration of stars or miasmas. After a real struggle that occurred not so many years ago, certain maladies were shown to be induced either by small animals or minute plants, *e.g.*, protozoa, fungi, bacteria and spirochetes. Indeed, the victory was so great that most workers in time began to consider that all infectious diseases, including those whose incitants had not been discovered, must be caused by agents similar to those already recognized. According to them, there could be no infections that were not caused by protozoa, fungi, bacteria or spirochetes, and to intimate that some infectious agents might be inanimate constituted heresy of the first order. Even at the present time, the cause of certain diseases is said by some individuals to be unknown or undiscovered, because no cultivable bacterium or visible protozoan parasite of etiological significance has been demonstrated in them. For instance, a few years ago Cowie made the statement in a scientific paper that the etiological agent of poliomyelitis is unknown, and in the recent book, *An American Doctor's Odyssey*, Heiser remarked that "the microbe which causes smallpox has never been discovered."

In spite of the general acceptance of the idea that all infectious diseases are caused by protozoa, fungi, bacteria or spirochetes, some workers have always contended that there might exist other infectious agents incapable of classification with those already known. Furthermore, very early in the bacteriological era a few discerning individuals appreciated the fact that there was no reason, except analogy, for assuming that all infectious agents must be living autonomous organisms. Through the activities

[1] Presidential address delivered before the Society of American Bacteriologists at its Thirty-eighth Annual Meeting, Indianapolis, Indiana, December 29, 1936.

of these investigators a group of disease-producing agents, known as viruses, has gradually become recognized. The exact nature of these agents is not known; some may be the midgets of the microbial universe, others may represent forms of life unfamiliar to us, while still others may be inanimate incitants of disease. Regardless of lack of complete knowledge of their nature, it is decidedly incorrect to say that these agents are unknown. The incitants of smallpox, vaccinia, poliomyelitis, yellow fever, fowl plague and tobacco mosaic are known; they can be recognized or identified in a variety of ways; they can be separated one from another and from other kinds of infectious agents; they can be used for extensive experiments conducted either *in vivo* or *in vitro*. Thus, to the initiate the term virus used in connection with an infectious agent has lost its old indefinite meaning and has acquired a new significance similar in exactness to that borne by the words bacterium and spirochete. The terms virus of smallpox, *Virus variolae, Virus myxomatosum* (Sanarelli) and virus of poliomyelitis are now as definitive as are the terms bacillus of typhoid, *Bacillus typhosus*, meningococcus and staphylococcus. Such a statement does not imply that all viruses are alike in nature and that a subdivision of the viral group is not essential. The proper time for this subdivision, however, has not yet arrived.

Microörganisms were known to exist long before their relation to disease was appreciated. After the discovery of this relation it was not uncommon for more than one kind of organism to be accredited with the ability of producing the same malady. This fact is not surprising in view of the almost universal distribution of microbes. As early as 1840, before the specific relation of microörganisms to disease was accepted, Jacob Henle stated the conditions that should be met before an agent could be considered the proved cause of an infectious malady. Unfortunately, investigators were not guided by Henle's remarks, and it was necessary for Robert Koch to restate and emphasize them 40 years later.

In an article on the etiology of tuberculosis Koch in 1884 made the following statement:

> The facts obtained in this manner can in every possible way serve as proof to which only extreme skepticism can still raise the objection

that the organisms found are not the cause but only concurrent phenomena of the disease. To be sure this objection often has a real justification and therefore it is not sufficient to establish only the concomitant occurrence of disease and parasite but the parasite must be shown to be the real cause. This can be done only by fully isolating the parasite from the body and all products of disease which might be considered as having a deleterious effect and producing the disease again with all its characteristics by the introduction of the isolated organisms into a normal host. (Author's translation.)

In 1890, speaking of bacteriological research before the Tenth International Congress of Medicine in Berlin, Koch expressed the same ideas in the following less mandatory manner:

However, if it can be proved: first that the parasite occurs in every case of the disease in question, and under circumstances which can account for the pathological changes and clinical course of the disease; secondly, that it occurs in no other disease as a fortuitous and nonpathogenic parasite; and thirdly, that it, after being fully isolated from the body and repeatedly grown in pure culture, can induce the disease anew; then the occurrence of the parasite in the disease can no longer be accidental, but in this case no other relation between it and the disease except that the parasite is the cause of the disease can be considered. (Author's translation.)

The above conditions laid down for the proof of the etiological relation of a microörganism to a disease constitute what are now known as Koch's postulates. His dictum has had a profound influence on workers investigating infectious maladies and for many years an infectious agent was not accepted as the cause of a disease unless the postulates had been satisfied. With the development of the science of immunology, however, immunological reactions added much to the knowledge of the specific relation of microbes to disease, and now it is possible to bring excellent evidence that an organism is the cause of a malady without the complete satisfaction of the postulates. In spite of this fact, there are certain workers who still refuse to agree that the cause of an infectious disease has been discovered unless all the conditions originally laid down by Koch have been met. This is particularly true regarding the viral maladies, the etiologi-

cal agents of which have not been cultivated on ordinary lifeless media.

It is unfortunate that so many workers have blindly followed the rules, because Koch himself quickly realized that in certain instances all the conditions could not be met, and in his paper before the Tenth International Congress of Medicine (1891), from which I have already quoted, the following statement occurs:

> The proof has been fulfilled in a number of diseases, anthrax, tuberculosis, tetanus, and many animal diseases, in particular for almost all the diseases which are infectious for animals. Furthermore, it has been shown that in all cases in which it has been possible to demonstrate the regular and exclusive presence of bacteria in an infectious disease, the parasites never behave as accidental saprophytes but in the manner in which well known pathogenic bacteria act. Therefore, we are justified in stating that if only the first two conditions of the rules of proof are fulfilled, *i.e.*, if the regular and exclusive occurrence of the parasite is demonstrated, the causal relationship between parasite and disease is validly established. In accordance with this hypothesis we must then consider as parasitic a number of diseases in which it has not yet been possible—or only in an incomplete manner—to infect experimental animals and to prove the third part of the rules. To these diseases belong typhoid fever, diphtheria, leprosy, relapsing fever, asiatic cholera. In this connection I must mention cholera particularly because the inclusion of this as a parasitic disease has been opposed with unusual stubbornness. All conceivable efforts have been made to deprive cholera organisms of their specific character but they have withstood all attacks triumphantly and one can accept it as a generally confirmed and firmly grounded fact that they are the cause of cholera. (Author's translation.)

At the time when they were formulated Koch's postulates were essential for the progress of knowledge of infectious diseases; but progress having left behind old rules requires new ones which some day without doubt will also be declared obsolete. Thus, in regard to certain diseases, particularly those caused by viruses, the blind adherence to Koch's postulates may act as a hindrance instead of an aid. For instance, the idea that an infectious malady can be caused only by the action of a single agent is

incorrect, and, if Shope had adhered to old ideas, he would never have discovered that swine influenza as it occurs in nature is caused by the combined or synergistic action of two agents, one a virus not cultivable on lifeless media, the other an ordinary hemophilic bacterium. Furthermore, it has been demonstrated that at least one natural disease of plants is induced by the combined action of two viruses, each of which has been obtained free from the other and when so obtained each produces a characteristic malady different from that caused by the synergistic action of the two agents.

The idea that an infectious agent must be cultivated in a pure state on lifeless media before it can be accepted as the proved cause of a disease has also hindered the investigations of certain maladies, inasmuch as it denies the existence of obligate parasitisms the most striking phenomenon of some infections, particularly those caused by viruses. Moreover, it ignores the possibility that certain viruses may be fabricated autocatalytically in living cells. One might say that the present-day method of propagating viruses in modified tissue cultures should be considered as taking the place of cultivation on lifeless media. I doubt whether the substitution is warranted, because the principles underlying the two methods of cultivation are radically different and Koch certainly did not have tissue-culture methods in mind when he proposed his rules of proof.

Koch's postulates are responsible for some odd conclusions regarding the cause of certain viral maladies. For example, a few investigators have claimed that streptococci are the inciting agent of poliomyelitis. Such claims, according to them, are based on the fact that Koch's rules have been satisfied. That is, streptococci have been found associated with the disease, they have been obtained in pure cultures from patients with the malady, they produce paralysis when injected into monkeys and rabbits, and they have been recovered in pure cultures from the experimental hosts. Furthermore, individuals recovering from poliomyelitis possess antibodies against the streptococci. To those unacquainted with the viral field and particularly to clinicians and bacteriologists unfamiliar with the pathological picture

of poliomyelitis, these claims seem valid. Consequently, they wonder why streptococci are not more generally accepted as the cause of infantile paralysis. The reason for lack of general acceptance is a simple one; the disease produced in the experimental animals is not poliomyelitis. Paralysis is not a characteristic sign of a single disease, and the pathological picture observed in the experimental hosts is quite different from that seen in human beings dead of infantile paralysis.

It is obvious that Koch's postulates have not been satisfied in viral diseases. Moreover, it is equally evident that proof of the etiological significance of viruses has been obtained without their satisfaction. Such a statement, however, does not imply that certain conditons do not have to be met before the specific relation of a virus to a disease is established. The conditions are: (*a*) A specific virus must be found associated with a disease with a degree of regularity. (*b*) The virus must be shown to occur in the sick individual not as an incidental or accidental finding but as the cause of the disease under investigation.

In many respects the conditions just stated for viral maladies are similar to those of Koch for the proof of the specific relation of bacteria to disease. Nevertheless, there are certain differences. In the first place, it is not obligatory to demonstrate the presence of a virus in every case of the disease produced by it. Secondly, the existence of virus carriers is recognized. Finally, it is not essential that a virus be grown on lifeless media or in modified tissue cultures.

How does one go about proving that a virus is the cause of a disease? Viruses, regardless of whether they are parasites or the fabrications of autocatalytic processes, are intimately associated with host cells and, therefore, should always be found at the proper time in specific lesions. In addition, viruses, as is the case with bacteria, may be found also in the blood stream, not necessarily multiplying there but appearing frequently only as a phenomenon of overflow from lesions in the tissues. With these facts in mind, tissues with lesions, exudate from such lesions, and blood are collected aseptically and inoculated into a susceptible experimental host of the same or different species. The

material should be free from ordinary microbes; if not, the microbes should be killed or removed in a proper manner, *e.g.*, by filtration. If the inoculated animals become sick or die in a characteristic manner, and, if the disease in them can be transmitted from animal to animal by means of inoculations with blood or emulsions of involved tissues free from ordinary microbes or rickettsiae, one is fairly confident that the malady in the experimental animals is induced by a virus. On the other hand, such findings do not necessarily indicate that the active agent was present in the original material used for inoculation of experimental hosts.

When a natural disease under investigation exhibits characteristic features, *e.g.*, paralysis or intracellular inclusions, they are sought for in the experimental malady. If one finds them, one is encouraged, but proof is still lacking that the virus operating in the experimental hosts was present in the material taken from the individual with the natural infection. Not infrequently several viruses produce the same clinical and pathological pictures, and at times the same virus does not induce similar changes in different hosts. Consequently, regardless of the disease picture produced in the experimental animals, one is still faced with the problem of demonstrating that the virus causing it was present in the material used for inoculation of the first group of animals.

Experimental animals are subject to viral diseases of their own which may be encountered with sufficient frequency to cause mistakes. In this connection, I can speak from experience. At one time I thought that I had transmitted varicella to rabbits, for, when material from varicella patients was injected into rabbits and serial testicular passages were made, a virus that produced lesions similar to those observed in cases of human varicella was regularly obtained. However, later work in my laboratory and in that of Swift, involving injections of the virus into human volunteers, neutralization tests, and the discovery of the virus in stock rabbits, demonstrated conclusively that the virus with which I was working, now known as virus III, does not cause varicella in human beings, but produces a specific disease peculiar

to rabbits. My experimental animals were already carrying an agent capable of inducing lesions similar to those seen in varicella; the virus was activated by the experimental procedures.

Another example of the necessity of proving that a virus comes from a certain source is that encountered in our recent work on lymphocytic choriomeningitis. In this instance, the problem arose because the virus, with which we were working and which we believed came from sick human beings, is frequently found in mice under natural conditions. Furthermore, monkeys and guinea pigs are occasionally naturally infected. We were able, however, to show that our stock mice were entirely free from infection with this active agent, and it immediately became highly probable that we had actually isolated our virus from patients.

In addition to the fact that animals are subject to their own viral diseases which sometimes lead to confusion in the course of experimental work, they may become accidentally contaminated with an alien virus being studied in the laboratory to which they are susceptible. For instance, rabbits are highly susceptible to vaccine virus, and, if in this host serial testicular or cerebral passages, initiated by sterile broth, are made in a laboratory where the active agent is under investigation, it is almost impossible to avoid picking up the virus. This fact, which I have demonstrated more than once for my own satisfaction, most likely accounts for the ease with which certain Japanese workers seem to have isolated from human beings what they consider the specific viruses of varicella, measles and scarlet fever. In any event, the descriptions of the actions of their viruses and the intracellular changes observed in tissues infected with them are what one would expect to find as the result of a vaccinal infection. Thus, when several viruses are being studied in a laboratory, proper precautions must be employed to prevent the contamination of materials and animals used for the isolation of a virus from a newly recognized disease or for obtaining proof that a virus causes a clinical entity well known for many years.

Having demonstrated that a virus was obtained from an individual ill of a certain disease, one must then prove that the agent was actually causing the malady instead of occurring fortuitously

or instead of inducing a complicating or coexisting infection. There is no reason why individuals with poliomyelitis may not at the same time be affected with fever blisters; yet the virus of fever blisters recoverable from the patients is not responsible for their paralysis. When faced with such a situation, knowledge of the regularity with which a virus is associated with a given malady is of great assistance; if its presence is fortuitous or if it is the cause of a coexisting infection, it should not only be found irregularly in patients with the disease under investigation but should also be encountered under other conditions.

To illustrate the point in question certain facts about the development of knowledge of epidemic encephalitis or Economo's disease will be reviewed. Levaditi recovered a virus from a few cases of this malady, demonstrated that it produced an encephalitis in rabbits under experimental conditions, and, in spite of the fact that intranuclear inclusions were found in the brains of the rabbits while none were observed in human material, concluded that he had discovered the cause of the newly recognized infection of man. It remained for Blanc to demonstrate that Levaditi's virus was identical with one discovered a number of years previously and shown to be the cause of fever blisters. When this fact became known investigators registered doubt as to the etiological significance of Levaditi's virus in epidemic encephalitis. That doubt should arise is natural, because from a large number of cases only a few strains of the virus, ordinarily easily established in rabbits, were recovered. Furthermore, fever blisters is a common disease of man and many workers realized that its causative agent might occasionally be encountered accidentally in patients suffering from one of a number of maladies. Indeed, Flexner and Amoss searched for herpetic virus in the spinal fluid of patients with a variety of diseases and were rewarded for their trouble by finding it in the fluid of an individual with syphilis of the central nervous system. As a result of a great deal of work, most investigators are now of the opinion that Levaditi's virus or herpetic virus is not the cause of epidemic encephalitis, even though it has been recovered occasionally from the brain or spinal fluid of patients with the malady.

Knowledge regarding the regularity with which a virus is associated with a disease may be highly important, but information concerning the presence of antibodies against the agent and the time of their appearance in the serum of patients is equally important as evidence of etiological significance of the virus. At the present time neutralizing antibodies are the most important, but complement-fixing antibodies, agglutinins and precipitins are being recognized more frequently in certain viral diseases and may eventually assume a significant place in experimental work on viruses.

Under at least two sets of conditions a virus of no etiological significance in certain diseases may occur in patients suffering from them. First, patients who have been affected previously by a viral disease continue as carriers after recovery to harbor the agent. Under such conditions they would possess antibodies against this virus at the beginning of their new illness as well as during convalescence. Secondly, it is conceivable that a virus might gain entrance into an individual and remain there only a short time causing little or no reaction. Under these circumstances, the virus, although capable of causing disease in experimental animals, would not incite the production of antibodies in the patients with the result that their serum would be devoid of antibodies both at the beginning and end of their illness. Some may doubt that this state of affairs occurs naturally. Nevertheless, it has been encountered not infrequently in experimental work.

If a virus is the actual cause of a disease, immune substances are usually absent from the patients' serum at the onset of illness and make their appearance during the period of recovery. However, this is not universally true, inasmuch as recovery sometimes takes place without the development of antibodies, and occasionally an individual possessing antibodies against a virus succumbs to a disease caused by it.

Although the absence of antibodies for a virus at the onset of an illness and their appearance later in the course of the disease or during convalescence constitute highly suggestive evidence that the virus is responsible for the malady, they alone should not be

accepted as incontrovertible proof that such is the case. The following example illustrates a striking exception to the rule. The Brown-Pearce tumor is a transplantable carcinoma of rabbits. Rabbits possessing no antibodies against virus III promptly and regularly develop them and become refractory to infection with the virus within two weeks after inoculation with the tumor. In view of these facts, one might on casual reflection conclude that virus III is the cause of the tumor. This is not true, however, because the virus alone does not cause the tumor, and the tumor freed from the virus does not produce in rabbits antibodies or resistance to the virus. In this case it is obvious that virus III, of no etiological significance so far as the tumor is concerned, is regularly carried in it, inciting the production of antibodies against itself in the carcinomatous animals.

To summarize, it can be said that the cause of viral diseases is known and that Koch's postulates as proposed by him do not have to be fulfilled in order to prove that a virus is the cause of a disease. However, the spirit of his rules of proof still holds in that a worker must demonstrate that a virus is not only associated with a disease but that it is actually the cause. The methods of doing this are different from the ones used by Koch but are equally efficient. At the present time, this is accomplished by the production with a degree of regularity of a transmissible infection in susceptible experimental hosts by means of inoculation of material, free from ordinary microbes or rickettsiae, obtained from patients with the natural disease, and by the demonstration through the use of proper controls and immunological studies described above that the virus was neither fortuitously present in the patients nor accidentally picked up in the experimental animals. Changes, notably the more extensive use of tissue-culture technics and serological reactions, will in the future undoubtedly occur in the methods of establishing the specific relation of viruses to disease; the number of changes will be limited only by the amount of ingenuity of investigators. To obtain the best results, however, this ingenuity must be tempered by the priceless attributes of common sense, proper training and sound reasoning.

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SCIENTIFIC PROCEEDINGS

THIRTY-EIGHTH ANNUAL MEETING OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

INDIANAPOLIS, IND., DECEMBER 28, 29 AND 30, 1936

Headquarters: Hotel Lincoln

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ABSTRACTS*

Report of the Committee on Bacteriological Technic: Progress during 1936. H. J. CONN, *Chairman*; J. H. BROWN, VICTOR BURKE, BARNETT COHEN, W. M. JENNISON, J. A. KENNEDY AND A. J. RIKER, *Members.*

The Manual of Methods for Pure Culture Study of Bacteria, which is in charge of this committee, has been considerably enlarged by the periodical revision of the leaflets of which it is composed. It now consists of 176 pages, the first edition (1923) having been only 48 pages in length.

As a result of this increase in size and the expense of handling a loose-leaf publication, it has been necessary to increase the price of the Manual slightly this year. This was done with some hesitation, because it was feared that the volume of sales might decrease. The opposite has been the case, however, as the number of copies sold this fall has been unusually large. In the first ten months of 1936, 43 more copies have been sold than during the entire year of 1935.

The leaflets revised in 1936 have been: II, Preparation of Media; IX, The Determination of H-ion Concentration; also a new Table of Contents, and a revised Index. These have been issued in the quarterly publication: *Pure Culture Study of Bacteria.*

The total profits from the committee's publication work turned in to the Society Treasury for 1935 were $409.45, exactly 25 per cent of the receipts. During the first ten months of 1936, the amount turned in has been $303.00. It is not expected at the time of writing this abstract (November 15) that the profits for 1936 will equal those for 1935. The reason is that last year the policy was begun of offering two-year subscriptions to *Pure Culture Study* at a lower rate than for a single year; the result was a tendency for receipts to be high the first year and low the second.

If sales of the Manual continue at their present volume, it is hoped to be able to offer higher quantity discounts on this publication when

* This number of the JOURNAL has been edited by the Chairman of the Program Committee. Authors of the abstracts in the Scientific Proceedings have not seen proof, due to restrictions of time imposed by the printing schedule.

ordered for class work. Teachers desiring it for such purposes are advised to write to the committee chairman concerning these discounts.

Actions Taken by the Second International Microbiological Congress in London, 1936, Regarding Bacteriological Nomenclature. R. ST. JOHN BROOKS AND ROBERT S. BREED, Lister Institute, London, England, and New York Agricultural Experiment Station, Geneva, New York.

A condensed report of the actions taken by the Second Microbiological Congress in regard to bacteriological nomenclature and classification will be given. This will include (1) a report of the final action in regard to the status of the genus *Bacillus* and its type species *Bacillus subtilis*, (2) the action taken regarding the classification of the genus *Salmonella*, (3) the action taken regarding the duplication of generic terms among *Protista*, (4) the action taken regarding the proposal that bacteriologists follow the practice fixed by the International Code of Zoological Nomenclature in regard to the non-capitalization of specific names, and (5) the appointment of a Committee to make a report on the best procedure to follow in regard to the abbreviation of generic names.

GENERAL BACTERIOLOGY

G1. The Growth of Bacteria, Yeasts, and Molds in a Strong Magnetic Field. M. W. JENNISON. Massachusetts Institute of Technology.

Experiments to determine certain gross effects, if any, of a strong magnetic field on the growth of microörganisms were carried out with some 25 species, including representative forms from 12 genera of bacteria, four genera of yeasts and four genera of molds.

Small petri dishes (50 x 10 mm.) containing nutrient agar or other suitable solid media were employed and the poured plates were inoculated at one spot in the center with a straight needle. Immediately upon inoculation three of the plates were stacked between the poles of an electromagnet and three more were used as controls. The intensity of the magnetic field was approximately 3000 gauss; this field was uniform over the entire surface of the agar, and in most experiments was perpendicular to the surface. Experimental plates were exposed for 48 hours or longer, at about 25°C.

The following characteristics of the organisms were compared on experimental and control plates kept at the same temperature for the same length of time: size of colony (indicative of rate of growth), mor-

phology of colony, size and shape of individual cells, reaction to the Gram stain, pigment and spore production, if any.

There was no observable effect of the magnetic field on the above characteristics. In a few cases organisms were picked from experimental plates after exposure, inoculated onto new plates, and exposed again as before, this being repeated two or three times. No differences were noted under these conditions. Organisms were also the same on experimental and control plates when the magnetic field was parallel to the surface of the agar, in the few cases investigated.

G2. The Growth Response of Yeast Exposed to Monochromatic Ultraviolet Radiation. ALEXANDER HOLLAENDER AND B. M. DUGGAR, University of Wisconsin.

The yeast employed in this work was *Saccharomyces cerevisiae* (Fleischmann's no. 53), and the stock cultures were grown on potato glucose agar buffered with calcium carbonate. In the preparation of a suspension of cells, the growth was washed from the agar surface with physiological salt solution, and dispersion was effected by shaking and by filtration through cotton. Samples of the suspension were then exposed to measured intensities of monochromatic radiation at γ2650 Å, with constant stirring. It was arranged that the intensities used would give survival values of the order of about 10 per cent. The organisms irradiation-"killed" by the exposures were found to react in a manner quite different from those heat-killed, as revealed by staining techniques. The surviving organisms, in a liquid nutrient medium show an initial growth stage followed by a definitely extended lag phase. In single or balanced salt solutions the controls show no growth and give the usual dying curve. On the contrary, the organisms surviving irradiation display, in the same salt solutions, a very definite increase in colony-forming cells, the increase being several times that characteristic of bacterial response.

G3. Some Effects of Ultraviolet Radiation on Bacteria. B. M. DUGGAR AND ALEXANDER HOLLAENDER, University of Wisconsin.

The lethal effects of ultraviolet radiation on *Escherichia coli* and *Serratia marcescens* suspended in non-absorbing (for ultraviolet) salt solution were studied in regard to intensity, wave length, age, and mode of treatment preceding, during, and after irradiation. From a population as large as twenty billion, a determination has been made of the energy required to kill a single cell.

The effects of sublethal doses of ultraviolet radiation were investigated by studying the growth curves of surviving organisms in nutrient media and their behavior in inorganic salt solutions. The surviving irradiated organisms showed definite increase during the lag phase followed by an extended resting phase. In each case the response was quantitative, with certain limitations appertaining to the initial growth phase. The fate of these organisms in inorganic salt solutions (single and balanced) revealed an initial increase of the number of individuals among those irradiated, with controls showing practically no increase. The effect of the irradiation is apparently in the cell itself and not in the exposure medium.

G4. The Constancy of the Electrophoretic Migration Velocity of Escherichia coli after Diversified Cultivation. RALPH P. TITTSLER, University of Rochester School of Medicine and Dentistry.

The electrophoretic migration velocity of *Escherichia coli*, when measured under strictly uniform conditions, was found to be constant regardless of the conditions of cultivation. It is evident, therefore, that the electrokinetic potential of the cells is an intrinsic characteristic which is not easily influenced.

In this study 71 different culture media, utilizing a wide range of ingredients under varied conditions and involving changes in the hydrogen-ion concentration and in the length of cultivation, were employed. The composition of the media was altered by changing the usual basic ingredients such as peptone, meat extract, fermentable and non-fermentable carbohydrates, etc., and by adding such substances as blood, inorganic salts, etc. Ten serial transfers at 24-hour intervals were made on almost every medium, the cells to be tested being taken from every other generation. The cells were washed thrice and resuspended in double distilled water before measurement to free them of metabolites and other extraneous substances.

G5. Determinations of Ultraviolet Light Absorption by Certain Bacteriophages. LESLIE A. SANDHOLZER, MARVIN MANN AND GEORGE PACKER BERRY, University of Rochester School of Medicine and Dentistry and College of Arts and Science.

Determinations of the absorption of ultraviolet light by three of Burnet's bacteriophages for *Escherichia coli*, C13, C16, and C36, were made by the method of matchpoint spectrophotometry. The absorption of the sterile medium, of a two per cent peptone (Bacto) water, and

of a non-lytic filtrate of a young culture of the test organism was also determined. Bacteriophage C13, purified by the modified Kligler-Olitzki technique previously reported, was also studied.

The data are presented as curves with the wave length plotted as a function of the photographic intensity. All of the curves, with the exception of that for the sterile medium, showed maxima between 2,600–2,700 Å. With the sterile medium, no such maximum occurred, but the curve was practically straight in the zone mentioned. Each bacteriophage was characteristic in the quantity of light absorbed. For purposes of comparison, the quantitative results are given in the usual terms of photographic intensity (photographic intensity = log $\left.\frac{1}{\text{transmission}}\right)$: C16, 1.4; non-lytic filtrate, 1.02; C36, 0.82; C13, 0.78; sterile medium, 0.42; purified C13, 0.2.

No correlation between lytic titer and absorption was found. Absorption was also independent of nitrogen content, which was 0.2 mgm. per 10 cc. for the purified bacteriophage, approximately 33 mgm. per 10 cc. for all the other preparations investigated. The interpretation of these findings must await further study.

G6. The Ultimate R Culture Phase of the Pneumococcus. LAWRANCE E. SHINN, The Western Pennsylvania Hospital Institute of Pathology.

What we style as the ultimate R form of the pneumococcus differs morphologically from Dawson's Rn in the following manner: Instead of forming long chains of streptococci interspersed with diphtheroidal elements at times, it is characterized by long, non-septate filaments which form two kinds of gonidia, both coccoidal in form. It is thus representative of what Mellon has described years ago as a fungoid stage for *Bacillus coli*, *Bacillus alkaligenes*, certain diphtheroids, etc.

The simpler of these gonidial types originates in the center of colonies undergoing gradual lysis; the more differentiated takes origin in the peripheral, actively growing portions. Under conditions that are as yet uncertain, the first gonidial form may stabilize and grow as such for a more or less indefinite period in very minute colonies. Yet it may be reverted to the R form at will, and thence to the conventional smooth and mucoid colony types. The involutionary status of the gonidium is thus abrogated.

The second type of gonidium is capable of differentiating into tetrad forms with a high degree of dormancy. Rarely this dormancy has been broken by heat shock, which results in a culture composed of tetrads

and diplococci. These still produce greening on blood agar, but are no longer bile soluble. As yet they have not been reverted to typical pneumococci. The biologic significance of this ultimate R form appears to be its inherent potentialities for transformations to already established pneumococcal phases, as well as to additional ones, perhaps.

G7. *Studies on Escherichia coli-mutabile in Relation to Lactose Utilization.* ANNA DEAN DULANEY AND CHARLES J. DEERE, Departments of Bacteriology and Chemistry, School of Biological Sciences, University of Tennessee, Memphis.

Strains of *Escherichia coli-mutabile* from babies suffering with diarrhea have been found to vary in the presence of lactose, raffinose, salicin, and sometimes, dulcitol. In all cases of acid formation in broths containing such sugars red variants were demonstrated.

Very small amounts of lactose (0.025 per cent) are effective in producing variation. The time of addition of lactose to a culture growing in nutrient broth influences the rate of utilization and quantity of sugar used very slightly, if at all. Lactose added at the beginning of growth or after intervals as long as 60 days produces similar results.

All strains have retained their variability after three years and no stable white forms have been obtained. Individual cells and colonies vary greatly in their potential ability to give rise to red descendants. So far only one red variant has reverted; this occurred after serial passage in succinate broth (Hershey and Bronfenbrenner).

In lactose broth the white form grows more slowly during the first 12 hours than in plain broth and no significant sugar utilization can be demonstrated.

A study of the enzyme activity of white and of red organisms grown in plain and one-per-cent lactose broth has been made. Cells were harvested from such media after growth periods of six to 48 hours, washed, treated with toluol, and later tested for enzyme activity. Preparations from both types of media have been found active. Increase in lactase, sugar utilization, formation of acid, and the appearance of red forms are closely correlated.

G8. *Bacterial Variation: Further Observations on Variant Forms of Bacillus megatherium.* HAZEL B. GILLESPIE AND LEO F. RETTGER, Yale University, New Haven, Connecticut.

Pointed cells which taper off gradually and occasionally thin down to long, hair-like tips develop in large numbers at the peripheries of microcolonies of *Bacillus megatherium* when this organism is grown on agar

blocks containing unusually small amounts of nutriment. Pointing commonly occurs at the distal end of the cell in its relationship to the colony. The tendency to taper off appears to be a response to an insufficient supply of nutriment. It is, of course, possible that other factors may also lead to the development of similar morphological types. This phase of the problem is at present under investigation. If heavy inocula are planted on dilute media very little development occurs and morphological variants do not appear. *Bacillus megatherium* will not reproduce on 1.5 per cent Difco agar in water, and cells planted on this medium soon die.

In a former paper the occurrence of long, uniformly twisted pairs of filaments was reported. Futher study has indicated that this twisting phenomenon is dependent upon purely mechanical factors, chief among which is a tendency toward slightly more rapid growth in one of two filaments which are still held together, after fission, by a very tough protoplasmic strand or "plasmodesmid."

The growth of clubbed cells on fresh agar blocks has been followed microscopically. Both the "handles" and the "heads" of the clubs elongate and segment into the so-called normal rod form. Rods which develop from the thickened "head" of the club are considerably broader than those which develop from its "handle," and they remain so for at least 15 or 20 generations. The thick cells show other characteristics which differentiate them from the thin cells which develop from club handles. For example, individual thick cells tend, as they grow, to twist themselves together. In clusters they resemble earthworms in a knotted mass.

Serially-made photomicrographs of living and developing cells will be shown to illustrate the types of variation described.

G9. The Chemical Analysis of Staphylococcus aureus and a Rough Variant. WESLEY M. CLARK, Columbia Breweries, Inc., Tacoma, Washington.

In a previous study by Hoffstadt and Clark, a smooth strain of *Staphylococcus aureus* and a rough variant were analyzed for their soluble specific substances. The present investigation of the same two strains of the organism involves fractionation by extraction methods and chemical analysis of the proteins obtained. Cold extractions were made using: ether, distilled water, acetate solution buffered at pH 4.0, phosphate solution at pH 6.5, ammonia at pH 8.6 and again at pH 11.0, and 0.1, 0.25, and 0.5 N NaOH. The smooth undissociated strain gave rise

to 15, and the rough variant to 14 fractions. From each was obtained: one phospholipid fraction, one albumin, three fractions of which had relatively low total but high dibasic nitrogen contents, seven metaprotein fractions, and a non-extractable residue. Two alcohol-soluble portions were obtained from the S organism, and one from the R strain.

Best extraction occurred at pH 7, with markedly decreased extraction on both sides of this point. The S form showed a second maximum extraction when N/10 NaOH was used, after which it fell off rapidly with further increase in alkalinity, while the R forms gave increasing extractions with increasing OH-ion concentrations. Percentage of nitrogen extracted rose from minimum at pH 6.5 to maximum with N/10 alkali, falling off thereafter. The S fractions showed generally higher percentages than the R fractions. Amide, dibasic and non-amino nitrogen were highest at low pH while humin and mono-amino nitrogen were higher at high pH. One S and one R fraction were found to have the same chemical composition.

G10. Is Escherichia coli a Derivative of Aerobacter aerogenes in the Human Body? M. P. HORWOOD AND R. A. WEBSTER, Massachusetts Institute of Technology.

Seven samples of the ileostomy discharge and four specimens of the rectal discharge from a male patient taken during a period of about eleven months were examined for colon-aerogenes organisms. During the tests, 101 colonies were isolated from the rectal discharges and 120 colonies from the ileostomy discharges. All the organisms from both sources gave acid and gas in glucose broth, lactose broth and brilliant green lactose peptone bile, two per cent. All of the cultures produced acid in litmus milk and often in sufficient amounts to produce curdling. All of the cultures likewise fermented glucose in glucose agar shake cultures. Nitrate broth was also uniformly reduced to nitrites in every instance.

Tests on differential media and microscopic appearance indicated that the 101 cultures from the rectal discharges were typical fecal strains of *Escherichia coli*.

Of the 120 cultures isolated from the ileostomy discharges, only 17 or 14.2 per cent produced indol; 63 or 52.5 per cent produced H_2S; 15 or 12.5 per cent gave a typical reaction for *Escherichia coli* on eosin methylene blue agar while 87.5 per cent gave the characteristic appearance of *Aerobacter aerogenes*; 10 or 9.2 per cent liquefied gelatin and 115 or 96 per cent fermented sucrose broth with the production of acid and gas.

Only 14 cultures or 11.7 per cent gave typical reactions for *Escherichia coli* in the methyl red, Voges-Proskauer, uric acid and sodium citrate tests, while 89.3 per cent of the cultures gave reactions typical of *Aerobacter aerogenes*. Only one of the latter gave a reaction typical of the *Citrobacter* or intermediate group. Of the 14 cultures that gave typical reactions for *Escherichia coli*, 13 were obtained from the last two ileostomy discharges. This represents 39.4 per cent of the cultures isolated on these two occasions from the ileostomy discharges. It is possible that a change in bacterial flora is taking place in the small intestine at this late stage following the ileostomy.

From the above results it would appear that the normal bacterial flora in the small intestine consists essentially of *Aerobacter aerogenes* but that following passage of the intestinal contents into the large intestine a change occurs which transforms *Aerobacter aerogenes* into *Escherichia coli*. It is possible, therefore, if this transformation is not complete, that *Aerobacter aerogenes* may have much greater sanitary significance than is accorded to it at present.

G11. The Gram-Negative Bacteroides of the Intestine. James E. Weiss and Leo F. Rettger, Department of Bacteriology, Yale University.

A morphological, cultural and serological study was made of 73 strains of Gram-negative, non-sporulating, obligate anaerobic intestinal organisms belonging to the group of the so-called genus *Bacteroides*.

These organisms were found to be the predominant bacteria in the intestine of most of the human adults supplying material for examination, and in some instances could be isolated from highly diluted fecal suspensions long after all other organisms had been diluted out. These observations are in harmony with those of Eggerth and Gagnon.

Cultural properties were quite variable. A classification is suggested which is based largely on serological reactions, supported by morphological characteristics. This system permitted separation of the strains studied into four distinct species or sub-groups.

G12. Application of Semisolid Media to the Study of the Clostridium butyricum Group. Robb Spalding Spray, West Virginia University Medical School, Morgantown.

Organisms of the *Clostridium butyricum* group are listed in Bergey's Manual of Determinative Bacteriology under 26 synonyms. The identity of many of these strains is lost in a confusion due to incomplete

description, to impure cultures, and particularly to a lack of the original cultures for corroborative study.

A group of strains of presumably definite history has been brought together, plus a group of unknown strains from various sources, and the methods of cultivation in semisolid media have been applied simultaneously to all of the strains.

It is observed: first, that these methods prove entirely suitable for the cultivation of organisms of this group; second, that this collection falls largely into certain fermentative types; third, that there appear numerous specific fermentative variants; and fourth, that the present names of certain "type" strains do not agree with the original descriptions.

It is hoped eventually, from this study, to establish certain representative types under proper names, and to make these available to others for comparative purposes.

G13. A Comparative Study of Bacillus bifermentans (Tissier and Martelly), Bacillus centrosporogenes (Hall) and Certain Closely Related Proteolytic Anaerobes. FRANCIS E. CLARK AND IVAN C. HALL. University of Colorado School of Medicine and Hospitals, Denver.

This study was undertaken better to establish the definitive and distinctive properties of certain proteolytic anaerobes. Thirty strains of *Bacillus bifermentans* and 45 of *Bacillus centrosporogenes* were subjected to complete morphologic, cultural, physiologic, and serologic comparisons. No completely non-motile strains of the former were found, and the differences encountered were not deemed sufficient to separate the two species; accordingly, the latter term, previously proposed by one of us upon discovering motile strains in this group, is withdrawn in favor of an emended description of *B. bifermentans*.

Within limits, the hydrogen ion concentration of the culture media could not be held accountable for loss of motility in aging cultures of *B. bifermentans*. Media favoring vigorous growth encouraged the production of more robust and more persistently motile vegetative rods. Incubation at temperatures below 37°C. prolonged the growth phase and the period of motility; in some cultures at 20°C., *B. bifermentans* remained motile as long as 96 hours after planting.

Zeissler's *Bacillus putrificus-tenuis* was found to be identical with *B. bifermentans*. *Bacillus nonfermentans* may consist of strains of *B. bifermentans* with dormant fermentative ability which can be unmasked by repeated transfers of young cultures. But *B. bifermentans* is clearly

distinct from *Bacillus sporogenes*, *Bacillus tyrosinogenes*, *Bacillus subterminalis*, and *Bacillus flabelliferum*.

No distinct morphologic or cultural differences were found between *B. bifermentans* and *Bacillus sordellii*, but the pathogenicity of *B. sordellii* prevents us from advocating their consolidation. However, evidence of antigenic relationship appeared in weak cross agglutination reactions and in the observation that serum from certain rabbits immunized against *B. bifermentans* conferred passive immunity upon guinea pigs against either whole cultures or toxic filtrates of *B. sordellii*. Normal rabbit serums or serums prepared against *B. sporogenes* or *B. subterminalis* had no such protective action. Furthermore, rabbits actively immunized to *B. bifermentans* withstood the injection of five to ten times the amount of *B. sordellii* culture fatal to normal control animals or animals actively immunized to *B. sporogenes* or *B. subterminalis*.

There was no cross agglutination between *B. bifermentans* and *B. sporogenes*, *B. tyrosinogenes*, *B. subterminalis*, or *B. flabelliferum*. With identical immunizing procedures, lower homologous agglutinating titers were obtained for *B. bifermentans* and *B. sordellii* than for *B. sporogenes* and *B. subterminalis*. Antigenic analyses showed that the somatic agglutinin titers for all four species were comparable but that the flagellar titers for *B. sporogenes* and *B. subterminalis* were much higher than those obtained for *B. bifermentans* and *B. sordellii*. This phenomenon is correlated with the more enduring motility of *B. sporogenes* and *B. subterminalis*.

G14. Biochemical and Serological Characteristics of Streptococci of Bovine Origin. W. N. Plastridge and S. E. Hartsell, Storrs Agricultural Experiment Station, Storrs, Connecticut.

Biochemical tests and the precipitin test of Lancefield were employed in determining the characteristics of streptococci obtained from freshly drawn milk samples.

Weakly hemolytic cultures associated with chronic bovine mastitis and possessing the biochemical characteristics of *Streptococcus agalactiae* were divided into three serological groups. The majority was placed in Lancefield's Group B, types I and II. The third group appeared to be serologically different from any of the Lancefield groups, although cross reactions were obtained with Lancefield's Group C and occasionally with Group B. No serological differences were noted between

cultures of *Streptococcus agalactiae* that fermented salicin and those that failed to do so. Several cultures which reduced methylene blue milk (1:5000) but otherwise resembled *Streptococcus agalactiae* were placed in Lancefield's Group B. In a few instances cultures which resembled *Streptococcus agalactiae* biochemically but not serologically, were obtained from mastitis-free quarters.

The majority of cultures placed in Group B of Plastridge and his associates, was divided into three serologically distinct types. No biochemical differences were noted between these three types. One of the three serological types was found to be identical with Lancefield's Group E.

Three beta-hemolytic cultures associated with acute mastitis were identical both serologically and biochemically with Lancefield's Group C. Extracts prepared from three beta-hemolytic cultures of *Streptococcus mastitidis* which were received from Engelbrecht reacted with antisera prepared against cultures representative of Lancefield's Group B type 1a.

The results obtained indicate that, while either biochemical or serological tests alone may give satisfactory results, both tests are necessary for the final identification of streptococci of bovine origin.

G15. The "Enterococcus" and "Lactic" Groups of the Streptococci. J. M. Sherman, Pauline Stark and E. S. Yawger, Jr., Cornell University.

The term "enterococcus group" is used to refer to *Streptococcus fecalis* and its related enterococci, *Streptococcus zymogenes, Streptococcus liquefaciens*, and other less known species. The "lactic group" includes *Streptococcus lactis* and its near relative, *Streptococcus cremoris*. The members of these groups resemble one another in gross morphological and cultural characteristics, in having low minimum temperatures of growth, and in having, in general, strong reducing action. These facts have led to the oft repeated statement that the two leading species of these groups, *Streptococcus fecalis* and *S. lactis*, are identical. The objects of this note are: (1) to define the enterococcus and lactic groups, and to differentiate them from each other; and, (2) to define *S. cremoris* as a species distinct from *S. lactis*.

The enterococci may be simply defined as those streptococci which have the ability to grow at both 10°C. and 45°C. So far as present knowledge goes, no other streptococcus has this combination of tem-

perature relationships, but it is desirable to further delimit the group. The members of the enterococcus group are able to grow in media containing 6.5 per cent sodium chloride, and in media having initial pH values of 9.6, properties not shared by other streptococci.

The streptococci belonging to the lactic group may be defined as those which are able to grow at 10°C. but not at 45°C., and which cause a complete reduction of litmus in milk, the reduction taking place before curdling. The litmus milk reaction is important since the *Leuconostoc* species, sometimes classified as streptococci, would mostly, if not entirely, fall in this temperature group.

S. cremoris differs from *S. lactis* by the inability of the former to produce ammonia in 4 per cent peptone; and its inability to grow: at 40°C., in the presence of 4 per cent sodium chloride, and in broth having a pH of 9.2. *S. cremoris* is also usually less tolerant to methylene blue than is *S. lactis*.

G16. The Value of the Temperature Limits of Growth in a Primary Grouping of the Streptococci. J. M. SHERMAN, Cornell University.

The temperature limits of growth of the streptococci were first used for taxonomic purposes in the differentiation of *Streptococcus lactis* from the mastitis type of streptococcus, and subsequent investigations have shown the value of growth temperatures in the characterization of other streptococci. For about ten years we have used the abilities to grow at relatively high and low temperatures, together with certain other important characters, as a means of grouping the streptococci. One of the values of this mode of approach is that it at once segregates many, *but not all*, of the non-pathogenic hemolytic streptococci which are found in such substances as milk, food products and feces.

In the grouping which follows, names for the respective divisions are used simply for convenience; some of these, especially "viridans," are probably not very apt. So far as the now known and adequately described streptococci are concerned, the growth temperatures appear to bring together somewhat related species into natural groups, but it is too much to expect that this will remain so true as more species become clearly defined.

For brevity, degrees Centigrade and growth are indicated by figures together with (+) or (−). Hemolytic properties are signified by (H+) or (H−). Instances in which species depart somewhat from the group type are indicated following the names.

"Pyogenic group" (10−, 45−, H+)

S. pyogenes (including *S. epidemicus*, *S. scarlatinae*, and *S. erysipelatis*), *S. mastitidis* (H±), and, probably, *S. equi* and the "animal pyogenes."

"Viridans group" (10−, 45+, H−)

S. salivarius (45±), *S. bovis*, *S. inulinaceus*, and *S. equinus.*

"Thermophilic group" (10−, 50+, H−)

S. thermophilus.

"Lactic group" (10+, 45−, H−)

S. lactis, and *S. cremoris.*

"Enterococcus group" (10+, 45+, H±)

S. fecalis (H−), *S. liquefaciens* (H−), *S. zymogenes* (H+), and at least one other less clearly defined hemolytic species.

G17. A Critical Study of the Fusobacterium Genus. II. Some Observations upon the Growth Requirements and upon Variation. E. H. SPAULDING AND LEO F. RETTGER, Department of Bacteriology and Immunology, Temple University Medical School, Philadelphia, and Laboratory of General Bacteriology, Yale University.

A classification of the fusiform bacilli into two groups has been proposed by us (J. Bact. 1936, **32**, 125). Since cell and colony morphology have a somewhat limited taxonomic value here, the division was based upon biochemical and serological reactions.

The proposed groups exhibit marked cultural differences which serve to explain the conflicting biochemical and cultural results reported by various investigators. The growth requirements of the two groups are of a widely divergent nature, one being markedly stimulated by fermentable carbohydrates, the other by added nitrogen compounds. A medium is recommended which is capable of supporting vigorous development of both groups. An accessory growth factor is essential for primary culture. The nutritive and anaerobic requirements, as well as the morphology, are altered markedly by continued artificial cultivation.

No evidence could be obtained that the spirillar variants are of common occurrence under laboratory conditions, although certain factors were discerned which favored the appearance of these forms.

An opaque mucoid variant is described. A granular type of growth in broth, which is also a manifestation of variation, has an important bearing upon the successful performance of biochemical and serological tests. All attempts to induce group or type transformation were unsuccessful.

Pathogenicity of certain strains was indicated, but no evidence of toxin production could be obtained.

G18. Pathogenicity and Classification of Pathogenic Yeasts. ERNEST A. PRIBRAM, Loyola University Medical School, Chicago.

The author demonstrated in a previous paper, that a classification of pathogenic bacteria, although based on a purely botanical system revealed, that the human and animal organism respond in a different way to different botanical units. Some of these units act on the myelogenous, other ones on the endothelial, others on the lymphatic system. Transition forms frequently combine the action on two of these systems. This paper considers in a similar way the classification of pathogenic yeasts and yeast-like microörganisms and the relationship of various genera to the types of lesions caused by them.

The localization of the infection is of less importance than the type of response. In meningeal and cerebral infection, there is no typical response, because of the softness of the tissue.

Attention should be called to a more detailed consideration of the connection between the histopathology and the classification of the pathogenic organisms.

G19. Classification of the Genus Monilia. DONALD S. MARTIN, C. P. JONES, K. F. YAO, and L. E. LEE, JR., Departments of Bacteriology and of Obstetrics and Gynecology, Duke Hospital, School of Medicine, Durham, North Carolina.

More than 150 strains of *Monilia* from human sources were studied and compared with strains obtained from Benham, Stovall, Langeron, Castellani and others. Colony formation on blood agar, type of growth on glucose broth, fermentation of various carbohydrates, microscopic morphology on corn meal agar, and serologic studies could be correlated sufficiently well to classify these organisms into six well defined groups. Consistent results with sugar fermentations could be obtained only after the organism had been passed through several generations on sugar free media. It was also found essential to cover the inoculated carbohydrate broth with a vaseline seal. Agglutinations with immune sera

were of some value in confirming the separation of these organisms into groups, but such a close antigenic relationship existed that it was not found practical to use this method as a diagnostic procedure. An outline of the procedures necessary for the identification and classification of this group of yeast-like organisms is presented. Only simple, easily prepared media are necessary and the technique described is easy to carry out.

G20. The Occurrence of a Hitherto Unrecorded Organism of the Genus Pseudomonas in Arizona. W. B. WEST, Arizona State Laboratory, Tucson.

A member of the genus *Pseudomonas* has been found in many water samples submitted to the Arizona State Laboratory for bacteriological analysis. It was noticed that when the organism was present in samples, great difficulty was experienced in confirming the presence of the colon-aerogenes group.

Studies were undertaken to identify the organism. Its distribution was studied and its possible significance is discussed.

Cultural difficulties encountered during identification suggest the possibility that other so-called species of the genus may be variant strains of this one species. The rough colony type appears to be the commonly occurring one, and is more stable during subsequent reculturing. The characteristics of this organism fail to agree with those of any reported species of the genus *Pseudomonas*.

The organism is found in seven per cent of all water samples received. It is found most frequently in summer. Most municipal and rural supplies have contained the organism from time to time. It appears to be bactericidal for the colon-aerogenes group and definitely reduces the possibility of recovering these organisms during the procedure of the Standard Methods test.

G21. Changes in the Infective Ability of Rhizobia and Phytomonas tumefaciens Induced by Culturing on Media Containing Glycine. B. J. LONGLEY, T. O. BERGE, JAMES M. VAN LANEN, and I. L. BALDWIN, University of Wisconsin.

When cultured on suitable laboratory media over long periods, strains of rhizobia and of *Phytomonas tumefaciens* normally exhibit little change in their ability to infect appropriate host plants. Cultivation of either of these organisms in media containing small concentrations (0.1 to 0.3 per cent) of glycine results in the partial or complete loss of infective

ability. Usually some ten culture generations in the glycine medium are sufficient to cause a partial or temporary loss in infectiveness. Further culturing (30 culture generations) on this medium usually results in a complete and permanent loss of infectiveness. Some strains of these organisms retain their infective ability, when subjected to such treatment, more tenaciously than do others.

Glycine is toxic to both organisms in low concentrations, and low concentrations must be employed at the start. Continued growth in the glycine media tends to develop a resistance, and larger concentrations can be employed after a few culture generations. When cultures are transferred from glycine media to the usual media, growth is at first slow. After two or three transfers on the usual culture media, the glycine-habituated cultures regain their normal rate of growth and resemble by all laboratory tests the original parent cultures. Such cultures, however, do not readily regain their infective ability.

Strains of *Rhizobium trifolii*, *Rhizobium phaseoli*, *Rhizobium japonicum* and *Rhizobium leguminosarum* behaved as indicated above. Strains of *Rhizobium meliloti* seem to be much less readily influenced by the presence of glycine in the medium. With the rhizobia, loss of effectiveness prior to loss of infectiveness, was frequently observed.

Alanine, glyclglycine and dicyanamide induced responses similar to glycine with several strains of *Phytomonas tumefaciens*.

G22. The Variable Properties of Potato as a Bacteriological Culture Medium. JANET McCARTER AND E. L. TATUM, University of Wisconsin.

The value of potato as a nutrient for bacteria varies with differences in the chemical composition of the potato. Media made from Wisconsin potatoes containing asparagin and from Idaho potatoes containing no asparagin were compared as to their suitability for the cultivation of human and bovine tubercle bacilli. Comparisons of the rate and the maximum amount of growth were made on potato slants moistened with nutrient broth and inoculated by flooding the surface of the slant with a drop of a suspension of the microörganisms; glycerine was added to the nutrient broth for the media upon which human tubercle bacilli were grown but not to the media for the bovine tubercle bacilli. The rate of growth was determined by gross observation and the amount of growth was determined from the dry weight of the cells harvested two weeks after growth had apparently ceased.

The growth on the Wisconsin and Idaho potatoes differed in rate and

in total amount: the human tubercle bacilli developed faster and slightly more profusely on the Wisconsin potato medium, while the bovine tubercle bacilli also at first grew more quickly on the Wisconsin potato medium but ultimately the amount of growth was about twice as great on the Idaho as on the Wisconsin potato medium. The absolute differences in the rates of growth on the two media varied with the amount of inoculum, being much more marked with smaller inocula.

However, asparagin was shown not to be the causative factor in the difference between the stimulating properties of the two kinds of potato, since the addition of asparagin to the medium made from the non-asparagin-containing Idaho potatoes failed to render it comparable to the asparagin-containing Wisconsin potato medium. Furthermore, a third medium made from a batch of Wisconsin potatoes *not* containing asparagin was found to be inferior to even the Idaho non-asparagin-containing potato medium for the growth of the human tubercle bacillus. Therefore, the causative factor is apparently a concomitant of asparagin. This factor may be present in the tryptophane-tyrosine fraction, which Tottingham and co-workers have shown to be increased in those Wisconsin potatoes which blacken upon boiling, since Tatum and co-workers have found that the presence of asparagin is associated with the blackening phenomenon also.

G23. Detection of Hydrogen Sulphide in Cultures. Charles A. Hunter, Max Feldman and Gilbert Crecelius, Laboratories of Bacteriology, University of South Dakota and State Health Laboratory, Vermillion.

Studies of ferric ammonium citrate and the soluble form of bismuth, such as bismuth liquor (3 grams bismuth citrate, 2 cc. of NH_4OH and 100 cc. of water), and bismuth and ammonium citrate Merck U. S. P. IX have shown that bismuth is more sensitive for the detection of hydrogen sulphide than iron. The hydrogen ion concentration has considerable influence on the sensitivity of iron, which has reacted more readily in an alkaline menstruum, while bismuth has been only very slightly affected by a change in reaction.

The use of either bismuth liquor (2.5 cc. per 100 cc. of medium) or 80 mgm. bismuth and ammonium citrate per 100 cc. of medium has proven to be much better than ferric ammonium citrate. Hydrogen sulphide has been detected in as short a period as 10 to 12 hours, and some organisms normally classified as non-hydrogen sulphide producers have given definite reaction in the bismuth medium.

Formulae for several media incorporating bismuth are presented and the methods of preparation are discussed.

G24. The Effect of Sodium Chloride on the Eh of Protogenous Media. L. S. STUART, AND LAWRENCE H. JAMES, Bureau of Chemistry and Soils, U. S. Department of Agriculture.

The effect on Eh of adding high concentrations of NaCl to protogenous culture media was studied using a platinum electrode with a 3.5 N KCl reference half cell. With higher initial pH values Eh was usually lower regardless of the amount of salt present. With pH values of 5.64 and lower, the addition of salt produced marked variations in Eh, while with initial pH values of 5.74 and above such variations were not as marked.

The steam sterilization of gelatine and peptone solutions containing high concentrations of salt caused marked oxidations.

The extent of oxidation caused by the addition of H_2O_2 to sterilized gelatine salt solutions depended upon the amount of oxidation previously induced by the combined action of the added salt and sterilization. The total amount of oxidation produced by steam sterilization in the presence of salt, followed by the addition of H_2O_2 was less than the total oxidation in the salt-free gelatine solutions, indicating a slight poising action by salt.

With the addition of a reducing agent (cysteine hydrochloride) to sterilized peptone solutions the total decrease in Eh was always slightly less in the presence of added salt regardless of concentration. This again suggests that salt adds poise to a medium.

G25. Comparison of the Germicidal Efficiency of Hypochlorites of High and Low Alkalinity. S. M. COSTIGAN, Research Department, General Laboratories Division, Pennsylvania Salt Manufacturing Company, Philadelphia.

Studies were made on the comparison of the germicidal efficiency of hypochlorite solutions of 200, 100 and 50 parts of available chlorine per million parts of water, prepared from hypochlorites of high and low alkalinity. Plate counts and growth in serial dilution tubes were compared to determine whether the dilutions of hypochlorite used killed the organisms or merely inhibited their growth.

To each 100 cc. of the various hypochlorite dilutions, a mixture of 9 cc. of a 24-hour broth culture of the organism and one cubic centimeter of sterile skim milk was added. At intervals of 3, 5 and 10 minutes, one

cubic centimeter of the disinfectant-organism mixture was run into sterile N/10 sodium thiosulphate blanks, one cubic centimeter into sterile distilled water blanks, and one cubic centimeter into each of five tubes of F. D. A. broth for serial dilution studies. The thiosulphate and water dilutions were plated on Bacto-nutrient agar. The plates and tubes were incubated for 48 hours at 37°C. before counts and readings were made.

The results show that even in the presence of high concentrations of organic matter a very close correlation exists between the plate counts and data from serial dilution tubes, indicating satisfactory killing and not inhibition. The strongly alkaline hypochlorite is more germicidal against the Gram-negative organisms than against the Gram-positive; however, the hypochlorite of low alkalinity is more germicidal against both the Gram-negative and Gram-positive organisms than the hypochlorite of high alkalinity.

G26. A New Method for the Evaluation of Germicidal Substances. A. J. SALLE, University of California, Berkeley.

It is customary to rate disinfectants on the basis of their phenol coefficients. The method is justifiable if the germicides are to be employed on skin surfaces or in connection with the sterilization of nonliving material. On the other hand, if the disinfectants are to be employed for internal administration or for the treatment of mucous surfaces, the above method presents very serious objections. It is usually considered advisable to use a germicide possessing a high phenol coefficient in preference to one less potent. It can be seen that there would be no advantage in using a compound with a phenol coefficient of ten on mucous surfaces if it possessed ten times the toxicity of phenol. Phenol could be employed to equal advantage with probably a saving in the cost of the disinfectant.

It is believed that a more accurate method of examining and rating germicides would be to test them for their effect on the growth of living embryonic tissue as well as for their ability to kill bacteria. A number known as the Toxicity Index is determined. The Toxicity Index is defined as the highest dilution of disinfectant required to prevent the growth of embryonic tissue divided by the highest dilution of the chemical required to kill the test organism. Theoretically, the smaller the Toxicity Index the more nearly perfect is the chemotherapeutic agent.

The compounds tested by this method fall into the following order on the basis of their Toxicity Indices: iodine, 0.09; iodine trichloride, 0.4;

mercuric chloride, 2.8; Hexylresorcinol, 3.0; Metaphen, 12.7; phenol, 12.9; potassium mercuric iodide, 13.3; Merthiolate, 35.3; and Mercurochrome, 262.0.

G27. The Efficiency of Commercial Chlorine-Containing Compounds Used in Various Cold Sterilization Procedures. DON C. LYONS, Jackson, Mich.

A study was made of a number of representative commercial compounds which are sold for the purpose of cold sterilization of glassware, etc., in restaurants, beverage dispensing establishments, and roadside stands. These compounds were of the type which bases its sterilizing or germicidal activity on the presence of available chlorine in compounds like calcium hypochlorite, sodium chloramide, etc. Rinse solutions when made up according to label directions are presumed to conform with state or local regulations concerning parts per million, chlorine to water.

It was found that there was considerable variation in the actual available chlorine in these compounds as analysed by the sodium thiosulfate test, or the orthotolidine test, and that, due to methods of marketing, many of the compounds are apparently worthless when they reach the consumers. Further, due to the relative unstability of the compounds or the rapid vaporization of the available chlorine after the packages are opened, many rinse solutions as made up are sub-standard or do not conform with regulations, although prepared according to package directions.

Change in merchandising or packaging of chlorine compounds is advised along with education as to the necessity of frequent changing of the solution. New products, which are stable and which can be tested quickly and easily for efficiency or germicidal activity, are needed for cold sterilization procedures.

G28. The Inhibition of Bacteria by Quinhydrone. DUDLEY PETERS GLICK AND LYNN L. GEE, Colorado State College, Fort Collins.

In studying oxidation-reduction potential as an index to bacterial activity during aging of ceramic clays, quinhydrone in 0.005 M concentration was employed to stabilize the potential of the system. When so treated, the shape of the E_h-time curves resembled that of similar curves plotted from unpoised clays, but at no time in the poised clays did the E_h attain the low level of the unpoised clays.

When three species of bacteria were treated in broth and on nutrient

agar by various concentrations of quinone, hydroquinone, and quinhydrone, quinone exerted the greatest inhibitive effect, while hydroquinone was least inhibitive. *Escherichia coli* was most easily inhibited and *Staphylococcus aureus* and *Bacillus subtilis* were little affected by concentrations in broth up to 0.006 M.

All of the species studied were inhibited by a crystal of quinhydrone or a drop of the saturated aqueous solution placed on heavily inoculated agar plates.

The inhibitive action of quinhydrone must be borne in mind whenever it is employed in E_h or pH measurements. Its use in control methods applied to dairy products, for example, would interfere with continued, proper microbial development. Wherever possible, the glass electrode should be substituted for the quinhydrone electrode in pH measurements.

G29. The Effect of Eh and Sodium Chloride Concentration on the Physiology of Halophilic Bacteria. L. S. STUART AND LAWRENCE H. JAMES, Bureau of Chemistry and Soils, U. S. Department of Agriculture.

Sarcina littoralis (Paulsen) was grown on media with varying percentages of added NaCl up to 27.5 per cent, over a predetermined range of *Eh* values. In media containing high concentrations of salt growth occurred only at relatively low *Eh* values, while in low-salt media growth occurred over a much wider range of *Eh*. In general, growth on high-salt media having low *Eh* values was characterized by pigment production and an inability to grow down into the medium itself, whereas growth on media of low salt content did not produce pigments and was uniformly distributed throughout the liquid substrate. Cellular morphology remained constant except for the development of smaller cells at the lower concentrations of salt. Intermediary concentrations of salt were less favorable for growth than either low or high concentrations.

Eh measurements made on incubated cultures indicated a marked oxidative metabolism for this organism when pigment was produced and a definite reducing metabolism when pigment was not produced.

G30. A Study of Some Oxidations and Reductions Concerned in Respiration of Bacteria. E. J. ORDAL AND H. O. HALVORSON, Department of Bacteriology and Immunology, University of Minnesota.

A study has been made of the enzymes of normal and variant strains of *Escherichia coli* which appear to be concerned in the production of gas from sugars or formic acid. It has been found that normal strains of *E. coli* have enzymes which can activate formate and hydrogen to reduce methylene blue, and also enzymes which can activate carbon dioxide to react with hydrogen and produce formic acid. In the variant strains of *E. coli* which are unable to form gas, one or more of these enzymes is lacking. Some variants are unable to activate hydrogen, others are unable to activate formic acid, and still others can activate both hydrogen and formic acid but appear unable to activate carbon dioxide. It would appear from our data that the enzymes that will activate formate, carbon dioxide and hydrogen are concerned in gas production by these organisms, and that if any one of these enzymes is lacking, the organism is not able to produce gas from sugars.

G31. Nitrogen Metabolism of Certain Colon Bacteria. P. L. CARPENTER AND W. H. PETERSON, University of Wisconsin, Madison.

The utilization of peptone and ammonia nitrogen in the presence of glucose by *Escherichia coli*, *Aerobacter aerogenes*, and an "intermediate" group has been studied by periodic determination of total soluble, amino and ammonia nitrogen. The *Escherichia* and *Aerobacter* strains utilized considerable amounts of ammonia nitrogen in the absence of organic sources. However, when organic nitrogen was present, there was no decrease in ammonia nitrogen, which indicates that organic nitrogen was the preferred form. Both organisms caused decreases in amino and soluble nitrogen, but the decrease in soluble nitrogen was more marked with *Aerobacter aerogenes* than with *Escherichia coli*. It is probable that the decreases in amino nitrogen and in soluble nitrogen are intimately associated through the hydrolysis of peptide linkages and subsequent utilization of the amino nitrogen formed.

The results with the "intermediate" bacteria were rather indefinite, although in general the organisms resembled *Escherichia coli* in their utilization of ammonia and peptone nitrogen.

G32. A Study of d-Arabinose Fermentation. STEWART A. KOSER AND ELIZABETH F. VAUGHAN, University of Chicago.

Previous work has shown that *d*-arabinose is subject to a delayed fermentation by many bacteria which attack the common *l*-form quite readily. A further study of the delayed fermentation of *d*-arabinose has been made. Increased concentrations of the sugar in broth, 2.0

and 5.0 per cent, did not accelerate the fermentative process. The use of shallow layers of medium to afford greater aeration gave no evidence of causing speedier splitting of the sugar. Anaerobic or partially anaerobic conditions did not appreciably hasten the fermentation. In a synthetic medium with *d*-arabinose as the only source of energy, most of the organisms were unable to initiate growth.

The slow fermentation was accelerated by successive transfers into *d*-arabinose broth. After ability to ferment the *d*-form readily had been acquired, no alteration in fermentative behaviour toward the *l*-form of the sugar or toward other common sugars was apparent. Also, this newly-acquired ability to ferment the *d*-sugar was retained by many cultures for periods up to two years, beyond which the tests were not carried. On *d*-arabinose agar plates daughter colonies or papillae containing rapidly-fermenting variants appeared within the original non-fermenting colonies.

When cells were removed from *d*-arabinose broth cultures prior to fermentation and transferred to new tubes of the same sugar medium, the fermentation occurred in the usual time. When filtrates of cultures, from which the cells had been removed, were reinoculated with cells which had not been in contact with the sugar, the fermentation was delayed. Thus, during the interval before fermentation the principal change seemed to be an alteration in the cells and not a conversion of the *d*-sugar to some more readily assimilable form before it was finally broken down.

G33. The Effect of Certain Colloids upon Endospore Formation by Bacillus subtilis. J. L. ROBERTS AND I. L. BALDWIN, University of Wisconsin.

Certain hydrophilic and hydrophobic colloids, when added to a Bacto-peptone basal medium, have been found to appreciably increase sporulation by *Bacillus subtilis*. Of the hydrophobic colloids, iron, silica and charcoal have been tested. Since the action of each of these seemed to be essentially the same, charcoal alone has been used extensively throughout the study. The addition of charcoal to 0.5 per cent Bacto-peptone water has been found to stimulate sporulation to from 200 to 300 per cent above the untreated peptone control. If the charcoal is removed by filtration prior to inoculation, the stimulation is somewhat greater. The amount of total nitrogen was in every case adjusted so as to be the same in both adsorbed and unadsorbed samples.

The results of Henrici, Williams and others indicate that in peptone

solutions, the amount of sporulation is inversely proportional to the amount of available nutriment. However, in the case of peptone adsorbed by charcoal, the amount of sporulation appears to be independent of the concentration of nutriment, since by evaporation to concentrations equivalent in total nitrogen to 1.5 per cent peptone, just as good sporulation was obtained as in 0.3 per cent concentrations. These results lead us to believe that some commercial peptones may contain a principle, removable by certain colloids, which is inhibitory to sporulation.

Hydrophilic colloids have been found to stimulate higher sporulation than the hydrophobes. Agar in amounts as small as 0.06 per cent is definitely stimulatory. The results of a series of experiments indicate that the action of agar is not due to its nutritive value, its action in bettering oxygen relationships, its electrical change, or its ability to hold metabolites within the vicinity of the cell. Agar which has been rendered hydrophobic by $MgSO_4$ or by alcohol, and subsequently washed, is stimulatory to approximately the same degree as charcoal, but not to the same degree as hydrophilic agar. Thus, while it appears likely that agar, like charcoal, may stimulate by virtue of adsorptive properties, there is evidence that some other property, as the hydrophilic characteristic itself, may be functional in stimulating sporulation.

G34. Antibiosis between Escherichia coli and Salmonella schottmülleri.
MacDonald Fulton, Biological Laboratory, Brown University and School of Medicine, Saint Louis University.

Growth curves of a strain of *Escherichia coli* and of *Salmonella schottmülleri* were studied in a synthetic medium. In mixed culture the sum of the growth of both strains, expressed in cell hours, was 53 per cent of that in the two controls. This depression was not proportionately divided between strains, coli giving 74 per cent as many cell hours in association as in pure culture, while schottmülleri gave only 35 per cent. The cell hours grown by schottmülleri were 54.5 per cent of the total cell hours of both pure cultures, while the corresponding figure for the mixed culture was 36 per cent. The difference, 18.5 per cent, represents the degree of inhibition of schottmülleri by coli during the growth cycle as a whole. The criteria of antibiotic action proposed by Nissle, Wikullil and Neufeld are not as adequate indications of such action as the cell-hour values for an association relative to its pure culture controls.

G35. Lysozyme Studies of Tissues from Animals Deficient in Vitamin A.
PAUL S. PRICKETT, NORMAN J. MILLER AND FRANCIS G. McDONALD, Bacteriological Laboratory, Mead Johnson and Co., Evansville, Indiana.

Vitamin A has been described as "anti-infective." The literature reports that animal tissues normally contain just enough excess of lysozyme to protect them against infection and that the lytic titer of tears is lowered in xerophthalmia, an A-deficiency lesion. Hence, it appears reasonable to assume that an A-deficient diet causes a lowered concentration of lysozyme in the tissues.

On this assumption, tissues from rats on an A-deficient diet and the corresponding tissues from rats on a normal, stock diet were titrated to compare their respective lysozyme contents. In addition, titrations of the tissues of rats on the A-deficient diet in which lysozyme fluid was substituted for the drinking water were compared to corresponding tissues from normal rats. The tissues (blood, eyes, lungs, liver, spleen and kidney) were dehydrated in acetone, and extracted in physiological saline. These extracts were titrated for their lysozyme content.

Contrary to our assumption, the lysozyme content of the A-deficient tissues was consistently higher except in the animals receiving lysozyme fluid instead of water, whose tissues had the same content as the normal animals' tissues. Thus it appears that: (1) lysozyme and vitamin A are independent factors in the rats' defense mechanism; (2) on a vitamin A-free diet the rat attempts to compensate for this deficiency of vitamin A by increasing the lysozyme content of its tissues; (3) when lysozyme fluid is substituted for drinking water with an A-deficient diet the rat is neither stimulated to produce excess amounts of lysozyme nor to store it in its tissues.

MEDICAL BACTERIOLOGY, IMMUNOLOGY AND COMPARATIVE PATHOLOGY

M1. The Animal Reservoirs of Brucellosis.
WILLIAM A. HAGAN, Department of Pathology and Bacteriology, New York State Veterinary College, Cornell University, Ithaca.

A review of our information about the animal reservoirs of the organisms of the *Brucella* group has been made. That human infections may and do occur as a result of contacts with cattle, goats and swine, and their secretions, is regarded as adequately proven. The possibility that horses, sheep, dogs, and fowls may act as sources of infection for

other species of animals and for man, is discussed. We have little reliable information on this matter; however there seems to be no doubt but that horses may sometimes act as such sources of infection. On the other hand it seems highly improbable that sheep, dogs and fowls ever play such a rôle, even though they may be invaded by *Brucella* and become reactors to the agglutination test for brucellosis.

M2. The Significance of the Horse in Brucellosis. CHARLES M. CARPENTER AND RUTH A. BOAK, University of Rochester School of Medicine and Dentistry.

Epidemiological studies of equine brucellosis indicate that the horse may be a more significant source of infection for man and cattle than is usually recognized. The examination of serum for abortus agglutinins from 347 horses, taken mostly from four stables, showed that 27 per cent had a titre of 1:25, 14 per cent a titre of 1:50, and eight per cent a titre of 1:100 or higher. Tests on a group of mares, repeated over a period of two years, disclosed that some of them had become infected without presenting clinical evidence of brucellosis. A few of the mares that developed high titres became temporarily sterile. Frequently the titre at the first test was 1:25, while at successive examinations it increased to 1:100 or higher, only to subside later to 1:25 or to disappear. These mares had been in contact with cattle with Bang's disease. Examination of the milk from five of the reacting mares that foaled normally revealed no evidence of *Brucella abortus* infection.

The serum from two of five horses with fistulous withers reacted at titres of 1:200 and 1:100, respectively, while the remaining three contained no *Brucella abortus* agglutinins. A bovine strain of *Brucella abortus* was isolated by guinea pig inoculation from the purulent exudate of one of these horses. Two children who had contact with this horse developed serious undulant fever.

M3. Brucella abortus in Horses. A. W. DEEM, Ohio State University, Columbus.

Abortus agglutination titers were determined on the blood sera of 189 horses, and a record was kept of the conditions for which they were treated. Thirty-four of these had either fistulous withers or poll-evil, or both. Of these 34 cases, 28 (82.3 per cent) gave positive reactions in serum dilutions of 1:50 or higher. Of the other 155 horses examined, 29 (18.7 per cent) had positive tests in the same range of serum dilutions. Of 103 apparently normal horses in the local field artillery unit,

ten (9.7 per cent) were positive for agglutinins in dilutions of 1:50 or 1:100, none higher.

By direct isolation we were able to obtain *Brucella abortus* in pure culture from nine of 15 samples of material obtained from as many previously unopened fistula cases, and from one of two specimens of synovial fluid in other bursitis conditions.

A simplified method for isolating *Brucella abortus* from otherwise uncontaminated material is given. We were unable to recover *Brucella abortus* from pus from any of five cases of fistulous withers from one to six weeks after they were opened.

M4. Human Infections with Brucella melitensis variety melitensis. ALICE C. EVANS, National Institute of Health, Washington, D. C.

The National Institute of Health is conducting surveys carried out by three investigators in different sections of the United States, to determine the prevalence of chronic brucellosis. Serums from suspected cases are sent to the laboratory at Washington for agglutination tests. They are also utilized in a study to determine the distribution of *Brucella melitensis* variety *melitensis* in this country.

A study of cultures is the most satisfactory method of identifying *Brucella* strains, but cultures are not readily obtainable from the chronic cases which are the subject of our present investigations.

Information also may be gained concerning the type of infection in any given case by a study of the patient's blood serum for agglutinin absorption reactions. This test differentiates the *Brucella* prevalent in this country into two groups: the abortus-suis, and the melitensis groups. The reliability of this test for identifying the type of the infecting strain can be estimated by a study of the literature. Out of 259 *Brucella* strains isolated in all parts of the United States and studied by various investigators for correlation between agglutinin absorption and bacteriostatic reactions, only 19 strains, or 7.3 per cent of the total number were atypical, with serological reactions contradicting the identification indicated by the bacteriostatic reactions. Ninety-two and seven-tenths per cent of strains agreed according to the two methods of grouping.

In order to carry out the agglutinin absorption test successfully, it is necessary to have a serum with a titer of one to 160 or higher—a titer not often maintained in chronic brucellosis. Hence the number of serums which have been available for the agglutinin absorption test has not been great.

From Bexar County, Texas, nine serums have been received with titers high enough for the test; six of them gave agglutinin absorption reactions agreeing with the melitensis type. From Mecklenburg County, North Carolina, seven suitable serums have been received and five of them gave reactions agreeing with the melitensis type. From Wyandotte County, Kansas, five suitable serums have been received, and two of them gave reactions agreeing with the melitensis type.

These results suggest that the *Brucella melitensis* variety *melitensis* is responsible for a considerable percentage of human infections in various parts of this country.

M5. The Chemical Constitution of the Endo-Antigen of Brucella Cells. ROBERT B. PENNELL AND I. FOREST HUDDLESON, Department of Bacteriology, Michigan State College.

A fraction, constituting approximately 25 per cent by weight of the dried cell, has been repeatedly isolated from organisms of the genus *Brucella.* It is responsible for the toxic action noticed upon intraperitoneal injection of *Brucella* cells into normal guinea pigs. The fraction precipitates immune serum in dilutions of from 1:400,000 to 1:1,000,000 and is actively antigenic.

Chemical analysis has shown the fraction to have the following constituents:

Acetone in the presence of HCl extracts a di-ketone. Ether and chloroform extract a fat, which yields upon saponification a fatty acid, melting at 20°C. to 30°C., and a solid alcohol which is not a sterol. Together these constitute about 15 per cent of the total fraction.

The nitrogen content varies from 6 to 8 per cent, of which about a third represents tryptophane nitrogen and the remainder is present as an unidentified crystalline substance.

Reducing sugars after hydrolysis represent 4 to 12 per cent of the fraction. Both a hexose and a pentose are present, tentatively identified from osazone preparations as glucose and arabinose, respectively.

Acetyl groups account for roughly 6 per cent of the fraction.

The remaining major portion of the substance yields upon hydrolysis an optically inactive hexonic acid and β-anthroquinone carboxylic acid or a homologue thereof.

M6. Serum Therapy in Human Brucellosis. LEE FOSHAY, General Hospital, Cincinnati, Ohio.

In terms of therapy the distinction between acute and chronic brucel-

losis in man is drawn arbitrarily at the end of the seventh month of disease. On this basis antiserum is given to acute cases. Chronic cases with acute severe exacerbations which of themselves may endanger life are given serum also. Cases of average severity receive 60.0 cc. of serum.

Antibrucella serum, from either goat or horse, was given in 77 consecutive acute cases for which complete quantitative data are available, with post-therapeutic observation periods of sufficient length to justify conclusions concerning permanent recoveries. Fifty-four patients received goat serum; 21 received horse serum. No differences attributable to animal source were noted. No patient failed to make a permanent recovery. The average duration of disease to serum administration was 7.6 weeks (minimum 1 week, maximum 29 weeks). The average duration of fever after serum was 16 days (minimum less than 1 day, maximum 90 days). The average duration of symptoms after serum was 29 days (minimum less than 1 day, maximum 210 days). The average disability period after serum was 1.7 months (minimum zero, maximum 9 months). The average total duration of disease was 3.9 months (minimum 1.2 months, maximum 11.0 months). The distribution of degree of severity of these infections was: 2 very severe, 27 severe, 44 moderate, 2 mild and 2 ambulatory.

Serum therapy in 10 chronic cases showed 6 satisfactory recoveries and 4 completely unsatisfactory outcomes (including one death).

M7. The Detection of Antigenic Variants of Brucella by Means of the Opsono-Cytophagic Test. Myrtle Munger, I. Forest Huddleson and Sherwood Wakeman, Department of Bacteriology, Michigan State College.

It has been found that there is a constant correlation between antigenic variation of strains of the species of *Brucella* and their susceptibility to phagocytosis by neutrophiles in normal whole blood. A strain of *Brucella* that is unstable to the action of heat or to basic fuchsin in a 1:4000 dilution does not make a satisfactory antigen for the agglutination test. Instability to heat and dye is not a fixed characteristic of many strains. The antigenic or unstable variants are phagocytised by neutrophiles in normal whole blood as well as in blood containing *Brucella* immune opsonins. Normal strains of *Brucella* are ingested by cells only when in the presence of specific immune opsonins.

The opsono-cytophagic test offers an accurate and simple means of detecting antigenic variants in the *Brucella* groups. The employment

of the opsono-cytophagic test for the detection of individuals susceptible or immune to *Brucella* infection requires the use of a normal culture.

M8. A Study of Brucella Infection and Immunity in a Large County Hospital. I. FOREST HUDDLESON, S. E. GOULD, MYRTLE MUNGER, AND DORIS M. PAULSON, Michigan State College and Eloise Hospital.

The study involved 8124 males and females. The subjects were divided into the four following groups, according to their status in the hospital: miscellaneous hospital patients, mental hospital patients, indigents, and food-handlers and employees.

The status of the subjects as regards *Brucella* infection, immunity and susceptibility was determined by the aid of the Brucellergin skin test, the agglutination and opsono-cytophagic tests, blood, stool and urine culture.

Of the total number examined, 10.3 per cent gave a positive Brucellergin test. Of the four groups examined, the highest incidence of infection, 10.5 per cent, was found among the mental patients and the highest incidence of immunity, 7.5 per cent, was found among the employees. Data are presented showing the comparative value of the serum agglutination test and the intradermal test in determining *Brucella* infection and immunity.

Data are presented which show the accuracy of the diagnostic tests on a large group of individuals retested after seven months.

M9. Growth Zones of the Brucella in Semi-solid Media. CLAUDE E. ZOBELL AND KARL F. MEYER, University of California.

When semi-solid nutrient agar is seeded with a dilute suspension of *Brucella abortus*, growth appears in a narrow zone 5 to 8 mm. below the surface of the medium, the zone of recently isolated cultures being narrowest and deepest. *Brucella melitensis* growth zones are 2 to 5 mm. below the surface and *Brucella suis* multiplies on the surface to a depth of 4 or 5 mm. Studies on 17 recently isolated *Brucella abortus* cultures supplemented by observations on 450 stock cultures of all strains show that the depth and extent of the growth zones can be controlled to a certain extent by carefully adjusting the oxidation-reduction potential and pH of the medium.

Besides having a specific effect, the hydrogen ion concentration influences both the oxidation-reduction potential and the carbon dioxide tension of the medium. Carbon dioxide is essential for the growth of

all of the *Brucella*, recently isolated *Brucella abortus* being most sensitive in this respect, but carbonate-containing media of proper pH can provide optimal conditions. While free oxygen is likewise required by the *Brucella*, its effect is most manifest upon the oxidation-reduction potential. By substituting sub-lethal quantities of various oxidizing and reducing agents, the oxygen tension tolerance can be extended over a wide range.

The growth zone phenomenon of the *Brucella* is of little value as a single differential test but when used in conjunction with other oxidation-reduction activities as well as inhibition by dyes, hydrogen sulfide production and agglutinin absorption tests, certain interesting relationships between the various types and transitional sub-groups are shown.

M10. Undulant Fever Agglutinins in Blood of One Hundred People Drinking Brucella-Infected Milk. W. R. CARROLL, University of Florida, Gainesville.

Opportunity for studying the possible incidence of undulant fever agglutinins in humans was afforded by a colony of sub-normal children whose dairy herd showed a high percentage of Bang's disease.

Good agglutination was obtained in the various patients in dilutions of their serum as follows: three in 1:300, one in 1:150, nine in 1:75, and 11 in 1:25, while 17 showed only feeble agglutination in the 1:25 dilution of their serum. Some swine kept on the same pasture with the infected cattle showed agglutination in low titers of their serum.

M11. Experimental Staphylococcus Arthritis in Rabbits. Bacteriological and Pathological Observations. MARJORIE B. PATTERSON AND ROBERT L. PRESTON, Departments of Pathology and Bacteriology and of Orthopedic Surgery, New York Post-Graduate Medical School and Hospital, Columbia University.

Advances in knowledge concerning the staphylococcus group of pathogenic bacteria, particularly the recognition of the various staphylococcus toxins, the production of staphylococcus antitoxin and the recognition of staphylococcus bacteriophages, have aroused an interest in further studies and a hope that some of this new knowledge may prove useful in the practical control of the disorders due to infection of man with staphylococci. The present study is concerned especially with experimental infection of the knee joint in rabbits induced by injection

of a small quantity of bacterial culture directly into the cavity of the joint.

In general the rabbits proved to be very susceptible to inoculation with virulent strains of staphylococcus isolated from human sources. Strains recovered from lesions of furunculosis or chronic osteomyelitis induced severe local suppuration and destruction of the joint, frequently associated with multiple abscesses in the viscera. Other strains from minor skin lesions or from fulminating staphylococcus sepsis produced comparatively mild joint lesions which were readily amenable to conservative medical and surgical treatment, but appeared to serve as a source of toxin elaboration resulting in severe toxic degeneration of the viscera, usually fatal. The animals seemed to resist better when the infected joint was immobilized after inoculation. When inoculations were made with a toxicogenic strain, local incision for drainage, local irrigation with bacteriophage and with antitoxic serum gave a satisfactory result in the knee joint, but intravenous injections with antitoxic serum and with bacteriophage seemed to exert very little effect upon the outcome of the general toxemia. An intermediate group of strains varied in pathogenicity from these two more clear-cut types, and produced a moderately severe toxemia with abscesses in but one of the vital organs.

M12. Experimental Staphylococcus Arthritis in Rabbits. Clinical Course and Requirements for Treatment. ROBERT L. PRESTON AND MARJORIE B. PATTERSON, Departments of Orthopedic Surgery and of Pathology and Bacteriology, New York Post-Graduate Medical School and Hospital, Columbia University.

A study of the clinical course and pathological findings in these experimental joint infections reveals a wide variance in the reaction of the animal to the infecting organism. The character of the lesions seems to depend upon the type of *Staphylococcus aureus* used to infect the joint. From the analysis of the clinical course and pathological findings it is possible to state the requirements of effective treatment. In the cases which have invasive joint lesions with multiple abscesses of the viscera, bones and joints, the important consideration is the prevention or inhibition of the spread of bacteria through the blood stream. Intravenous specific bacteriophage is indicated. The cases which have no evidence of bacteriemia, no metastatic abscesses, or no tendency for local joint lesions to invade the bones or soft parts but have marked toxic degeneration of the viscera require specific antitoxin. All types

have severe anaemia and require blood transfusions. It is especially important that the cases infected with the toxic type of *Staphylococcus aureus* receive blood from donors which have a high staphylococcus antitoxin titre of their blood. It is emphasized that acute staphylococcic suppurative arthritis is a systemic disease with death frequently occurring during the first two weeks of infection, and therefore, the chief therapeutic indication at the onset is treatment of the generalized toxemia or bacteriemia rather than mere local joint incision. The type of treatment indicated varies according to the reaction of the animal to the particular type of *Staphylococcus aureus* responsible for the infection.

M 13. The Standardization of Staphylococcus Toxic Products. FLOYD C. TURNER, U. S. Public Health Service, Boston, Mass.

Soon after staphylococcus toxin, antitoxin and toxoid came to the attention of practicing physicians, the manufacturers of biological products prepared those substances, but as there was no national standard, both the physicians and the manufacturers were uncertain as to the proper potencies and dosages of the new products. The manufacturers volunteered and gave every possible aid in the establishment of a national standard. First, an international standard staphylococcus antitoxin was prepared and a unit was officially designated. The procedure was analogous to the establishment of diphtheria and tetanus antitoxin units. A United States standard staphylococcus antitoxin unit was then established which equalled in potency the international standard. A United States control staphylococcus toxin was made. Certain unsolved difficulties have barred the establishment of a standard for staphylococcus toxoid. Such information as was available has been presented to manufacturers and interested workers as a guide as to what a potent and safe toxoid should be, according to our present knowledge.

M 14. A Study of the Effects of Staphylococcus Exotoxin on Leucocytes and Bone Marrow of the Rabbit. A. L. JOYNER, R. H. RIGDON AND ROBERT HARE, Departments of Bacteriology and Pathology, Duke University School of Medicine.

Filtrates of broth cultures of *Staphylococcus aureus* were tested *in vitro* for the presence of leucocidin. A filtrate containing the potent leucotoxic factor was injected into normal rabbits. A temporary drop in the total white cell count was observed, a shift to the left in the differential picture and a succeeding hyperplasia of the bone marrow.

It is suggested that the toxin is a strong factor in the production of a similar picture in the human subject frequently observed in severe staphylococcus infections.

M15. The Production of Staphylococcal Antibodies in Human Subjects by the Injection of Staphylococcus Toxin. EARL L. BURKY, Wilmer Institute of Ophthalmology, Johns Hopkins University and Hospital, Baltimore.

Staphylococcus toxin, lethal for rabbits, but deficient in dermo-necrotic and hemolytic factors produces in human subjects, when injected intracutaneously (1) a rise in the complement-fixing antibodies, and (2) the ability to neutralize the lethal factor in rabbits. It does not produce a marked change in the dermo-necrotic and hemolytic antibodies normally present. Unexplained variations in the antibody content during the course of immunization have been described. Immune human serum combined with toxin has been used in the treatment of a case of inoperable osteomyelitis of the spine with complete recovery.

M16. Antitoxic Immunity in the Prophylaxis and Treatment of Staphylococcal Infections. C. E. DOLMAN, Connaught Laboratories (Western Division), Vancouver, Canada.

Laboratory and clinical evidence is presented to support the contention that the symptomatology of staphylococcal infection is attributable to liberation of specific soluble toxins. Antitoxic immunity, passively conferred or actively provoked, will protect certain laboratory animals against doses of living staphylococci lethal to non-immunized animals. Clinical response to staphylococcus toxoid or antitoxic therapy depends largely upon the provocation of an adequate titre of specific circulating antitoxin. A close parallelism does not always obtain between the degree of immunity and the titre of circulating antitoxin. Immunity, whether actively or passively acquired, depends not only upon the final titre of circulating antitoxin, but also upon the relationship between the titre of antitoxin in the blood and in the tissue cells, upon the rate of excretion of antitoxin therefrom, and upon the frequency and degree of exposure to contact with staphylococci. Special difficulties are encountered in immunizing against localized staphylococcal infections, owing to the heavy reinfection hazard represented by a ubiquitous microörganism. In the treatment of generalized staphylococcal infections, prompt administration of antitoxin is of paramount

importance, in order that the rapidly formed and rapidly acting toxins may be neutralized before fatal damage has occurred.

Owing to the peculiar complexity of staphylococcus toxins, it would seem that efforts should be made to develop criteria for the proper selection of strains for the production of toxoid and antitoxin. The true significance of staphylococcal antitoxic immunity should not be obscured before it has yet been learned, by advocating the use of mixtures containing some form of bacterial antigen in addition to the toxoid.

M17. The Immunological Specificity of Staphylococci. L. A. JULIANELLE, Oscar Johnson Institute, Washington University School of Medicine, St. Louis, Missouri.

The results of the studies on the specificity of staphylococci indicate the existence of at least two immunological types which are definable, not by agglutination, but by precipitation of the respective carbohydrates extracted from the organisms. The one type, A, is composed of strains isolated from pathogenic conditions, while the other type, B, comprises strains from non-pathogenic sources. The specific carbohydrates are differentiated chemically, by optical rotation and by the simple sugars resulting from hydrolytic cleavage. The specificity of the carbohydrates is proportional to the degree of hydrolysis.

In contradistinction to the carbohydrates, the nucleoproteins of staphylococci are species-specific and participate in common serological reactions.

Skin reactions elicited by the carbohydrates are almost entirely to Type A, the reactions occurring in few normal infants and children but reaching a maximum frequency of 65 to 70 per cent in normal adults. In these instances no precipitins accompany the skin reactivity. Patients recovering from infection also exhibit a skin reactivity to Type A carbohydrate, but only those suffering from generalized or severe prolonged infections elaborate specific precipitins.

Attempts to separate the types by a more rapid and practical method indicate that lysis by bacteriophage is not satisfactory. While the study is still in progress, it appears at the present time that Type A strains ferment mannitol while Type B strains do not.

M18. Experimental Staphylococcus Food Poisoning in Thirty-two Human Volunteers, G. M. DACK AND WM. E. CARY, University of Chicago.

The problem of establishing the staphylococcus as the cause of a particular outbreak of food poisoning is complicated by the fact that we have no simple method for determining which strains are of the food poisoning type. No laboratory animal has been found which is as susceptible as man when fed filtrates or cultures containing the enterotoxic substance from food poisoning strains of staphylococci. The present study is based on our experience in the past six years of feeding 32 human volunteers with foods experimentally contaminated with staphylococci or with filtrates prepared from veal infusion broth cultures. Twelve of these volunteers were fed several times. The incubation period was three hours or less in 15 out of 20 subjects who were made ill, four hours in three and five hours in the remaining two. Four volunteers had bloody vomitus and three passed bloody mucous stools. Moderately severe abdominal distress was present in most cases. The acute symptoms usually lasted from three to eight hours, but in some of the more severe cases diarrhea persisted for several days. Prostration was marked in 10 cases. In no subject where repeated feedings were made did one attack confer immunity to subsequent attacks. There was a marked difference in the susceptibility of volunteers. In one individual there was no reaction with a dose which produced a severe reaction in another. The mild symptoms occurring in some of the volunteers consisted of one or more of the following: obstipation, abdominal distress, vomiting without diarrhea, and diarrhea without vomiting. This experimental study is of value from the epidemiological standpoint in differentiating staphylococcus food poisoning from that due to other causes.

M19. A Food Poisoning Outbreak Due to Staphylococcus. WILLIAM B. McCASTLINE, R. THOMPSON, AND M. L. ISAACS, Columbia University.

An outbreak of food poisoning involving 31 persons occurred in a college dormitory. The source of infection apparently was an ice cream custard. Bacteriological examination of the two flavors served revealed total counts of four and ten billion, respectively. In each case staphylococci composed about 99.5 per cent of the organisms. The staphylococci were of two types. The first was orange, produced coagulase and soluble hemolysin and contained Julianelle's carbohydrate A, but did not ferment mannitol. The second strain differed from the first only in lack of orange pigment and in its ability to ferment mannitol. Throat and nose cultures of all workers concerned in the

preparation of the custard were made. Cultures identical (so far as our tests could determine) with the two strains predominating in the ice cream were isolated from two of the workers.

Growth curves of the organisms in a milk, egg and sugar mixture resembling the custard indicated that with an inoculum of 100,000 per cubic centimeter (an almost inconceivably large figure) seven hours would have been necessary to reach the numbers found in the original ice cream. The process of making the ice cream could have permitted a maximum of four hours incubation prior to freezing. Pasteurization studies indicated that the organisms are not unusually heat resistant. At 145°F. for 30 minutes, there is a reduction in numbers of 99.999 per cent. It must be noted, however, that the number of organisms surviving pasteurization is considerable when the initial numbers are as large as were found in the ice cream.

M20. Antigenic and Biochemical Properties of Staphylococci. RICHARD THOMPSON AND DEVORAH KHORAZO, College of Physicians and Surgeons, Columbia University, New York.

Antigens prepared from 271 strains of staphylococci from several sources were tested for precipitation with immune sera against nine of the strains. The organisms were found to fall into five groups: Group 1 (A), 78 strains, including Julianelle's Type A; Group 2 (B), 29 strains, including Julianelle's Type B; Group 3 (C), 64 strains; Group 4 (BC), 15 strains, precipitated by both 2 and 3 sera; and Group 5, 85 strains, whose antigens were not precipitated by any serum.

The 271 strains were tested for pigment production, fermentation of mannitol, production of coagulase and production of a soluble hemotoxin for rabbits' erythrocytes. There were marked differences between Group 1 and the other groups in regard to those properties. Ninety-three per cent of the strains in this group produced soluble hemotoxin and 75 per cent of the strains possessed all four of the properties tested for. On the other hand, about 95 per cent of the strains in Groups 2, 3 and 4 possessed none of the properties and only one strain in these combined groups gave positive results in all four tests. Group 5 occupied an intermediate position; 63 per cent of the strains showed entirely negative reactions; between seven and 24 per cent reacted positively to one or more of the tests and five strains (6 per cent) were positive for all properties.

In contrast to the marked correlation of soluble hemotoxin production with the presence of antigen A, visible hemolysis of rabbits'

blood plates was produced by 50 per cent or more of strains in all antigenic groups.

A definite correlation of antigenic grouping with the sources of the cultures was evident. Seventy-three per cent of strains from infectious processes fell into Group 1. Fifty per cent of strains from normal conjunctivae, noses and throats were in Groups 2, 3 and 4 with 21 and 28 per cent in Groups 1 and 5, respectively. Seventy-three per cent of 24 strains isolated from the air in various locations were in Group 5.

This work gives support to Julianelle's claim that "pathogenic" staphylococci possess a specific carbohydrate (A). It suggests that at least one group in addition to his Group "B" is present among the "non-pathogenic" staphylococci of the mucous membranes.

M21. The Action of Aldehydes on Purified Diphtheria Toxin. MONROE D. EATON, Department of Bacteriology and Immunology, Washington University School of Medicine, St. Louis.

Certain definite physico-chemical conditions are necessary for the modification of highly purified diphtheria toxin to toxoid by aldehydes and aldehyde compounds. These differ somewhat from the conditions for the modification of crude toxin because peptones and other substances in crude filtrates may react with aldehydes.

When purified toxin is incubated with 0.3 per cent formaldehyde at a pH of 8.6, detoxification occurs but there is also a loss or impairment of flocculating, immunizing, and combining properties indicating destruction of the toxoid. However, if the formaldehyde acts on toxin at pH 6.2 instead of at 8.6 a toxoid having unimpaired antigenic and flocculating properties results. Controls containing no aldehyde remain toxic.

One per cent hexamethylenetetramine at any pH from 6.0 to 9.0 modifies toxin to toxoid without destruction of its antigenic properties. Detoxification by this substance is much more rapid in acid than in alkaline solutions. Acetaldehyde ammonia modifies toxin to toxoid at pH 7.8, but very slowly or not at all at pH 6.5. Acetaldehyde sodium bisulphite and formaldehyde sulphoxylate produce a much slower modification than the corresponding aldehyde ammonia compounds. Aldol detoxifies but also destroys the antigenic properties. Glucose in an alkaline solution modifies purified toxin to toxoid thus resembling other aldehydes.

Possible mechanisms of the action of aldehydes on toxin, and the

rôle of ammonia, and amino-compounds such as peptones, in the toxin-toxoid modification will be discussed.

M22. The Antitoxic Titers of Human Subjects Following Immunization with Combined Diphtheria and Tetanus Toxoid, Alum Precipitated. F. G. JONES AND JAMES M. MOSS, The Lilly Research Laboratories and Indiana University School of Medicine, Indianapolis.

The power of the body to produce specific antibodies after the injection of several antigens simultaneously has been established by Gay and by others. In our work the use of combined diphtheria and tetanus toxoids, alum precipitated, in both animal and human subjects has shown that each toxoid acts independently and effectively in the production of its respective antitoxin.

In a group of human subjects, two injections of 0.5 cc. each were administered subcutaneously with a two-month interval between injections. By the Schick test and titration of diphtheria antitoxin content of the blood serum of each individual, the diphtheria antitoxin content was demonstrated to remain quite consistent over a six month period. The reduction in tetanus antitoxin titer after six months was practically the same as in individuals who received tetanus alum precipitated toxoid alone.

M23. Studies on Tetanus Toxoid. II. The Response of Human Subjects to an Injection of Tetanus Toxoid or Tetanus Alum Precipitated Toxoid One Year after Immunization. F. G. JONES AND JAMES M. MOSS, The Lilly Research Laboratories and Indiana University School of Medicine, Indianapolis.

Following two injections of tetanus alum precipitated toxoid or three injections of unmodified tetanus toxoid in 58 medical students, each subject developed some immunity. Immunity developed more rapidly in those treated with alum precipitated toxoid.

After six weeks, the average potency of the persons immunized was approximately 0.2 unit per cubic centimeter of serum; at 12 weeks it was 0.13 unit; at six months, 0.11 unit; and at 12 months, 0.06 unit.

Another injection of toxoid was administered to these subjects one year after the original immunization. At the end of one week the average potency showed an increase to 1.3 units per cubic centimeter and after four weeks to 3.3 units. Each subject gave a definite response with an average increase in potency of 22 times one week after injection and 55 times four weeks later.

M24. Growth and Toxin Production of the Diphtheria Bacillus in Synthetic Medium: the Effect of Inorganic Salts and Carbohydrates. MARY W. WHEELER AND LAURA MENDEZ, Division of Laboratories and Research, New York State Department of Health, Albany.

The effect of inorganic salts and carbohydrates on the growth and toxin production of the diphtheria bacillus in synthetic medium was studied. Magnesium, phosphorous, sodium, and potassium ions were found necessary for adequate growth. Within certain limitations, growth was not affected by increasing the quantities of either sodium or potassium, providing they were both present in sufficient quantities; but toxin production was greater in a medium which had a high sodium content than in a medium in which most of the sodium was replaced by potassium. Calcium was not essential for growth, but it was for toxin production. Toxin production was also dependent upon the sources of energy present in the medium.

M25. Hemolytic Streptococcus Toxins and Antitoxins. V. Titration by the Flocculation Reaction. LEO RANE AND LOUISE WYMAN, Antitoxin and Vaccine Laboratory, Massachusetts Department of Public Health, Boston.

A flocculation test for the titration of hemolytic streptococcus toxins and antitoxins has been developed which is comparable to the Ramon method for titrating diphtheria toxins and antitoxins. The technic employed in flocculation is essentially that of the Ramon test.

Concentrated toxins, prepared by the method described, are preferable to crude toxins because they flocculate with greater rapidity.

One unit of antitoxin will combine in the flocculation reaction with 60,000 S.T.D. of toxin instead of with the 50 S.T.D. to be expected on the basis of the definition of an *in vivo* unit of antitoxin. This relationship has been true of all toxins tested except those modified by formalin.

Streptococcus toxins and antitoxins display individual variations in their flocculating time and are influenced by the same factors as diphtheria toxins and antitoxins.

The flocculating antibody is produced in horses in amounts which parallel the antibody produced against the erythrogenic toxin. It is possible that the two antibodies are identical. The average of the *in vivo/in vitro* ratios was found to be 0.963. The correlation coefficient of the values obtained by the flocculation test and by the intracutaneous test in rabbits was 0.947 $\pm$0.023.

The Dochez NY 5 strain of hemolytic streptococcus was used as the basis for this study although flocculation was produced with other strains of hemolytic streptococci and their homologous antitoxins. The question of multiplicity of streptococcus toxins is not dealt with in this report.

M26. The Isolation of Pathogenic Staphylococci from Mixed Cultures. LILLIAN G. CURCIO AND GEORGE H. CHAPMAN, Clinical Research Laboratory, New York, N. Y.

Because hemolysis (aureus strains), coagulase and crystal violet agar reactions of staphylococci give results parallel with pathogenicity for rabbits, these reactions were used as *in vitro* indicators of probable pathogenicity. On proteose lactose agar (pH 10.0) containing 0.017 per cent brom-thymol-blue, positive strains grew luxuriantly while negative strains grew poorly; the results were parallel with 156 of 171 strains. This was considered a satisfactory correlation since the error of each *in vitro* reaction and of careful animal inoculation tests is estimated at about eight per cent. Confirmation of the growths by transplanting them to crystal-violet agar reduced the apparent error to 3.3 per cent.

Swabs from the nasal and oral cavities of persons with chronic illness were plated on brom-thymol-blue agar and growths were confirmed on crystal-violet agar. Duplicate swabs were plated on rabbit blood agar and the different types of colonies were isolated, purified and tested by *in vitro* methods. The results were similar with 73 of 81 pairs. In five instances an occasional *in vitro* positive colony was obtained from rabbit blood agar but not from brom-thymol-blue agar. In three instances a moderate growth of *in vitro* positive strains was recovered from brom-thymol-blue agar but not from rabbit-blood agar.

If the specificity of the brom-thymol-blue agar method should be confirmed by a more extensive series of tests, it would be a valuable medium for the isolation and quantitative estimation of probable pathogenic staphylococci.

M27. A Method for Studying the Concentration of Tubercle Bacilli from Sputum and Body Fluids. JOHN H. HANKS AND HAROLD F. CLARK, Department of Bacteriology, George Washington University Medical School and U. S. Public Health Service, Washington, D. C.

Since suspensions of tubercle bacilli in water could not be completely sedimented by centrifugation at high speed for several hours, it appeared that the collection of tubercle bacilli from sputum and body fluids by this method must be inefficient. The supernatant fluids which are usually discarded, following the NaOH digestion of tuberculous sputa and the centrifugation at high speed for 30 minutes, gave alum precipitates which often contained more bacilli than the original sediment.

At present the sputum is digested with an equal volume of normal NaOH and centrifuged at 500 times gravity for five minutes to remove the débris. The supernatant fluid is decanted into a clean tube from which samples are removed for study. Two methods of collecting the bacilli have been found most efficient and are now being compared. *Method A.* Add 0.1 cc. of 1 per cent ferric chloride to each cubic centimeter of digested sputum. *Method B.* Add 0.1 cc. of 5 per cent potassium alum to each cubic centimeter of digested sputum and then add 2.5 N HCl drop by drop with shaking until sharp flocculation occurs (at yellow-green to brom cresol green). These precipitates are packed by centrifugation at 500 times gravity for five minutes, transferred to glass slides with a platinum spatula, and smeared densely and evenly as in the Breed method. For quantitative studies the sediment is diluted to one-tenth the original volume of the sample, handled in calibrated capillary pipettes, and smeared on a measured area of slide. The smears are stained by the Ziehl-Neelsen method. Precipitates which reveal no tubercle bacilli after examination of 300 or more microscopic fields are being compared with Petroff's concentration method by means of cultivation and guinea pig inoculation. The general application of such methods to the collection of viruses and bacteria for many purposes is worthy of further study.

M28. The Bacteriology of Experimental Tuberculosis in Guinea Pigs. Bacteremia. M. H. Soule, Hygienic Laboratory, University of Michigan.

Cultures were prepared and stains were made of the blood of 326 normal healthy guinea pigs and 114 with advanced tuberculosis. In a later study, specimens were withdrawn from 62 heavily infected animals just before and 48 hours subsequent to the intradermal injection of tuberculin (O.T., 1.0 mgm.).

The method of cultivation was that of Löwenstein (Inter. J. Leprosy

1, 39–44 (1933)). The smears were made of the washed precipitate obtained from 1 cc. specimens of blood that had been discharged directly into centrifuge tubes containing 5 cc. of 2 per cent acetic acid and were stained by the Ziehl-Neelsen technique. Each film was examined by three trained workers.

A general as well as a local reaction followed the introduction of the tuberculin and there was a significant increase in the percentage of positive blood cultures and positive blood films from specimens taken during this period.

M29. The Isolation of Acid-Fast Bacteria from Soil. RUTH E. GORDON AND WILLIAM A. HAGAN, Department of Pathology and Bacteriology, New York State Veterinary College, Cornell University, Ithaca.

Frey and Hagan have demonstrated that acid-fast organisms are common in soil. Their method of isolating these organisms involved the use of a very high incubation temperature (47.5°C.) to eliminate overgrowths with molds. This procedure is quite successful but, obviously, only those organisms capable of growing at unusually high temperatures are recovered. Tests on a series of strains of saprophytic acid-fast bacteria in our collection showed that many would not grow at that temperature. It seemed probable that the method succeeded only in isolating those forms that were thermoduric and missed those that were not. The present paper deals with a technic by which it has been possible to isolate, rather easily, a number of strains that could not have been isolated with the technic of Frey and Hagan.

Utilizing the observation that many of the saprophytic acid-fast bacteria are able to develop in a synthetic medium which is essentially devoid of nitrogen, whereas in this medium molds failed to develop, it was possible to develop from soil inocula, cultures which in nearly every instance contained acid-fasts and other bacteria but were free from molds. From this medium enrichment of the acid-fasts was accomplished by transfers into the paraffin medium of Söhngen. Here most of the organisms that had grown along with the acid-fasts in the first medium failed to develop, and it was easily possible to make isolations by plating on a synthetic medium. Sixty strains were isolated from 45 consecutive soil samples. Of these, 27, or nearly half, would not grow at 47.5°C., and would have been missed in the earlier technic.

M30. Cultivation of Vaccinia Virus in the Rabbit Fetus. ORAM C. WOOLPERT AND FRED W. GALLAGHER, Department of Bacteriology, Ohio State University, Columbus.

Although the rabbit is a classic test animal for vaccinia virus, no studies are on record in which the susceptibility of the rabbit fetus to this virus has been determined directly. By a technic previously described, fetal rabbits, from 21 to 24 days of age, have been inoculated with the Levaditi strain of vaccinia, and with a chick membrane strain supplied by The Eli Lilly Company. The fetuses were removed by cesarean section, 3 to 5 days after inoculation.

In appropriate dilution the virus was found to produce characteristic gross lesions in many organs, particularly the skin, lung, kidney and liver. Larger amounts of the virus killed the fetuses in one to three days. Titration of the virus content of fetal tissues in adult rabbit skin frequently gave positive results at 1/100,000 and occasionally at 1/1,000,000 dilution. There seemed to be a correlation between the presence of gross lesions and the virus titre.

The virus has been carried through five passages in rabbit fetuses without apparent modification of its biologic properties, the fetal skin being used in each case as the source of subinoculation material.

M31. The Duran-Reynals Factor in Fetal and Adult Guinea Pig Tissues and Its Possible Bearing on Fetal Susceptibility to Vaccinia Virus. N. PAUL HUDSON AND OLIVER N. FELLOWES, Department of Bacteriology, Ohio State University, Columbus.

In studies reported by Woolpert and by Stritar and Hudson, the guinea pig fetus was found to be more susceptible to vaccinia virus than is the adult. Duran-Reynals and others have demonstrated that when mixtures of certain tissue extracts and viruses or bacteria are inoculated into susceptible animals, the experimental group shows an increase in titer of the infectious agent and a greater spread of the lesions than does the control group. On this basis, a difference in the concentration of the Duran-Reynals factor in fetal and adult tissues might be a factor in the relatively greater susceptibility of the fetus to vaccinia virus.

Saline extracts were made of various organs from normal 35- to 40-day-old guinea pig fetuses and from the same organs of the adult of the same species. These various extracts were added to ten-fold dilutions of vaccinia virus, and the mixtures incubated and inoculated intradermally into the depilated backs of white rabbits. Measurements and

character of the lesions, and the titers of virus controls and virus-organ extract mixtures were recorded and compared. From the measurements, individual and total areas of the virus control and the virus-organ extract lesions were calculated for the fetal and adult series, and ratios determined.

The ratios between controls and organ extract areas in the fetal and the adult series were almost identical. From the data obtained, the concentration of the Duran-Reynals factor in fetal guinea pig tissues is approximately the same as that in the adult guinea pig tissues. Therefore, the Duran-Reynals factor does not appear to be the sole basis for the greater susceptibility of the fetus than of the adult to vaccinia virus.

M32. Permeability of the Blood-Central Nervous System Barrier to Certain Antibodies in Monkeys Infected with Poliomyelitis. HOWARD J. SHAUGHNESSY, THOMAS C. GRUBB AND PAUL H. HARMON, Division of Laboratories, Illinois Department of Public Health and Department of Surgery, University of Chicago.

To determine whether or not the blood—C.N.S. barrier is permeable to certain antibodies injected during or before the preparalytic stage of experimental poliomyelitis, some 30 monkeys (*Macacus rhesus*) were injected with hemolysin (sheep erythrocyte amboceptor), human convalescent serum, diphtheria antitoxin or typhoid antiserum. Samples of blood and spinal fluid, obtained by lumbar or cisternal punctures, were collected during the preparalytic and paralytic stages of the disease. At autopsy portions of the spinal cord and choroid plexus were removed and tested for their antibody content.

The presence of antibodies in the specimens of blood, spinal fluid, spinal cord and choroid plexus was determined by the following tests: hemolysin by the usual hemolytic system; convalescent serum by precipitin tests using an antiserum obtained by injecting rabbits with convalescent serum; diphtheria antitoxin by intradermal guinea pig neutralization tests; and typhoid antiserum by the agglutination test.

The results indicated that the intravenously injected antibody could rarely be detected in the cerebrospinal fluid. In the very few instances in which the antibody was detected, it was present in very small amounts. These findings suggest that poliomyelitis does not have a "meningeal" stage, since it is known that the barrier is permeable to antibodies in a true meningitis caused by other infectious agents. The implications of these studies in the treatment of poliomyelitis are discussed.

M33. The Tick as a Vector for the Virus Disease, Equine Encephalomyelitis. JEROME T. SYVERTON AND GEORGE PACKER BERRY, University of Rochester, School of Medicine and Dentistry.

As we have already reported, a new experimental host, the "gopher," *Citellus richardsonii* (Sabine), has been found for the virus of equine encephalomyelitis. This host is representative of a large group of closely related rodents native to the United States. It may be significant that these rodents, the disease equine encephalomyelitis, and ticks of the genus *Dermacentor* have the same geographical and seasonal occurrence. With this in mind, we present evidence that the tick, *Dermacentor andersoni* (Stiles), may well serve as a natural vector in the dissemination of the virus of equine encephalomyelitis, Western strain.

Adult and nymph ticks were allowed to engorge on separate guinea pigs immediately after the pigs had been inoculated with brain tissue-equine encephalomyelitis virus suspension. At intervals of from 32 to 80 days thereafter, successive stages in the developmental cycle of the tick, larvae, nymphs and adults, were fed on different groups of normal gophers or guinea pigs. Infection resulted in these animals. Continuity of the virus through all of the stages in the developmental cycle of the tick, including survival through the egg stage, has been demonstrated. So far, this period of continuity has lasted for 130 days.

As far as we are aware, this is the first time that a tick of the genus *Dermacentor* has been implicated in the transmission of a filterable virus disease. Further studies which include other species of ticks, and which involve tick transmission of the virus to horses are in progress.

M34. Cultivation of the Virus of St. Louis Encephalitis. R. W. HARRISON AND ELIZABETH MOORE, Washington University, St. Louis.

Three strains of the virus of St. Louis encephalitis have been cultivated *in vitro*. Media consisted of finely minced tissue from mouse and chicken embryos suspended in Tyrode's solution and in rabbit serum diluted with Tyrode's solution. Cultures were transplanted serially over a period of four months. Presence of the virus in cultures was tested at frequent intervals by intracerebral inoculation of mice. The clinical signs and pathological changes in this animal were typical of St. Louis encephalitis.

The virus was not recovered from cultures to which immune rabbit serum had been added.

Cultivation of the virus through a number of generations in developing chick embryos was also accomplished. Although the pathological lesion was confined to the chorio-allantoic membranes, virus was recovered from brain, liver and spleen as well as from the membranes of the embryo. Microscopic lesions were found, however, in the brains of some of the chicks which were hatched after inoculation. Mice were infected with tissue suspensions from each egg passage generation and in addition with brain emulsion from a few chicks infected as embryos. Young chicks were also found to be susceptible to the virus inoculated intracerebrally.

M35. Further Studies on the Relationship of Streptococci to the Viruses of Encephalitis and Poliomyelitis. EDWARD C. ROSENOW, Mayo Foundation, Rochester, Minnesota.

The constant presence of the pleomorphic streptococcus having peculiar neurotropic and other properties in encephalitis and poliomyelitis, the presence of small diplococci, stainable by a special method, in filtrates of these viruses, and many other facts, suggest a relationship between this organism and the viruses of these diseases. Proof of this relationship has been attained through the use of a medium consisting of infantile tissue, one in which acid reaction by the growth of the streptococcus is prevented, i.e., chick-mash medium. This is prepared by macerating with a meat-chopper the 19-day chick, shell and all. To this mash from three to seven parts of distilled water are added; it is then tubed, autoclaved and layered with liquid petrolatum. The streptococcus grows rapidly in this medium, and as the cultures become old, exceedingly small forms appear and many disappear entirely. Filtrates of "old" cultures of the streptococcus in this medium, far removed from the original source, commonly yield virus takes in mice, guinea pigs and rabbits. As the experimentally produced virus is passed successively through animals the streptococcus appears and disappears in cycles of from three to five or more passages. Takes in monkeys have been obtained consistently with virus derived from streptococci having neurotropic virulence and cataphoretic velocity, after the virulence of the virus had been increased by successive passage through mice, guinea pigs or rabbits. After the primary take in monkeys of the experimental virus, as in the case of natural virus, little difficulty was encountered in passage from monkey to monkey. The symptoms which begin after a period of incubation, usually of from three to seven days, are in the first takes chiefly "encephalitic" but on

successive passage tend to become more "poliomyelitic." The microscopic lesions resemble closely those of encephalitis and poliomyelitis. The disease so obtained causes a high mortality. Several monkeys which had recovered from poliomyelitis following inoculation of "natural" virus resisted inoculations of highly virulent experimental virus. A practical method for the immunization of monkeys is under study.

M36. The Production of Immunity with Purified Suspensions of the Rabies Virus. C. A. BEHRENS, L. B. SCHWEIGER, AND J. L. REEVES, Purdue University, Lafayette, Indiana.

Vaccines used in prophylaxis against hydrophobia contain not only the virus of rabies but a great deal of nervous tissue. Over 60 per cent of the protein can be removed from suspensions of the fixed virus by precipitating the tissue at its iso-electric point.

When by the addition of weak organic acids, varying H-ion concentrations are obtained, the resulting preparations are either turbid, containing much of the tissue or water-clear and devoid of most of the foreign protein.

The preparation of homogeneous emulsions of rabic tissue is stressed. The minimum lethal dose of these preparations is 0.5 cc. of a 1:22,000 dilution injected intracranially. Such emulsions were treated with one hundredth normal citric acid until the desired pH was obtained, centrifuged and the supernatant fluid removed. The virus was rendered avirulent by acidification to a pH 4.9 to 5.2 by the further addition of citric acid and incubation at 25°C. for 24 hours. Then, tenth normal sodium carbonate was added until pH 7.2 was reached.

Rabbits inoculated subcutaneously or intravenously with suspensions of the avirulent virus of varying degrees of purification are able to withstand as high as four minimum lethal doses.

M37. Studies on the Cultivation of Dermacentroxenus rickettsi in Vitro. IDA A. BENGTSON, National Institute of Health, Washington, D. C.

The rickettsiae of Rocky Mountain spotted fever have been cultured through several series of transplants in modified Maitland media, one series having been transplanted through 12 successive generations. For culture media one part of fresh guinea pig serum was combined with four parts of Tyrode or Baker solution, and to this was added minced chorio-allantoic membrane of 13- or 14-day old chick embryos. Growth has been obtained at both 37°C. and 30°C. A greater multiplication of

rickettsiae was obtained as the result of the substitution of Baker solution for Tyrode solution.

The rickettsiae are present in the cytoplasm of the cells of the chorio-allantoic membrane as well as in certain undifferentiated tissue. In smears they appear most numerous at the degenerated margins of the tissue particles. They present a somewhat variable morphology, occurring as slender bacillary bodies and shorter coccoidal forms, singly, in pairs and occasionally in chains. Guinea pigs inoculated with cultures develop typical temperature reactions and scrotal lesions followed by death in a high percentage of the animals.

M38. Further Studies on Bacterium necrophorum Isolated from Cases of Chronic Ulcerative Colitis. G. M. Dack, Lester R. Dragstedt, and Theodore E. Heinz, University of Chicago.

Bacterium necrophorum has been commonly found associated with ulceration of the colon in man and monkeys. In chronic ulcerative colitis in man it has been found in enormous numbers in the lesions cultured at proctoscopic examination. It outnumbers other organisms in the severely diseased isolated colons of patients upon whom ileostomies have been performed. It has not been recovered when the bowel heals. From our studies it appears to be present in the bowel, since it readily appears when necrotic lesions are present in the colon regardless of the inciting cause, i.e., bacillary dysentery, trauma, etc.

Bact. necrophorum does not appear to be a clearly defined bacterial species and has received many different names. It is similar if not identical to what is called in the literature *Bacillus funduliformis.*

M39. The Type-Specific Antigen of Streptococcus hemolyticus (Group A). Stuart Mudd, Edward J. Czarnetzky, Horace Pettit, David Lackman and Earl W. Flosdorf, School of Medicine, University of Pennsylvania.

A labile, type-specific, nitrogen-containing compound has been prepared from each of eight types of Group A hemolytic streptococci. This compound has been isolated from the soluble products of hemolytic streptococci disintegrated by each of three methods: (*a*) sonic vibration, (*b*) drying by the lyophile process and grinding under liquid air, and (*c*) lyophile processing and grinding in a ball-mill cooled in a Dry-Ice bath.

Absorption of homologous antiserum with labile substance completely removes the type-specific mouse-protective, phagocytosis-promoting,

agglutinating and precipitating antibody. Much evidence has been obtained to indicate that the labile substance is Griffith's typing agglutinogen, and that Lancefield's M substance is a derivative of the labile substance. The labile substance is present in full activity in mucoid forms and in reduced activity in glossy variants.

Type 1 labile substance may be dissolved in 70 per cent alcohol and recovered without losing its capacity of combining with antibody. It has been rendered inactive, however, by: (*a*) storage for a few days in the refrigerator, (*b*) treatment with 1 per cent formaldehyde for 30 minutes, (*c*) exposure to pH 10.0 for 10 minutes, (*d*) heating at 56°C. for 30 minutes, and (*e*) digestion for three hours in a 5 per cent solution of Northrop's crystalline pepsin. The labile substance passes through a Berkefeld filter and can be preserved by the lyophile process. Its antigenicity is under investigation.

A hemolysin has been isolated by one of us (E. J. C.) from β-hemolytic streptococci of various Lancefield groups. Even very dilute solutions lyse red blood cells. This hemolysin contains nitrogen and phosphorus in the ratio of three molecules to one, and its molecular weight has been determined as 2260.

M40. Extraction of the Labile Components of Streptococci by Physical Means. EDWARD J. CZARNETZKY, DAVID LACKMAN, HORACE PETTIT, C. H. SHAW AND STUART MUDD, School of Medicine, University of Pennsylvania.

Disruption of hemolytic streptococci by physical means has led to isolation of a labile type-specific antigen in a state of activity not attained by earlier methods of extraction. Three such methods have been developed: (*a*) disruption by vibrations of audible frequency, (*b*) by grinding the lyophile-processed cells under liquid air, and (*c*) by grinding the lyophilized cells in a special ball-mill cooled externally in a Dry-Ice bath.

Each of these three methods presents certain advantages and disadvantages. In the sonic method, no preliminary lyophile processing is required, but the original cost of installing a suitable vibrator is high. The liquid air method is efficient, but dangerous on account of explosions which might result from contact of organic material with a high concentration of oxygen. In this method the cells tend to be scattered by the rapid volatilization of the liquid air. The Dry-Ice method is efficient, but like the liquid air method requires preliminary lyophile processing of the bacterial cells.

In any of these three methods the bacterial cells are not subjected to high temperatures, chemical action, or conditions which are usually associated with denaturation or chemical reactivity.

An important labile component of hemolytic streptococci has been isolated from solutions obtained by each of these methods, and appears to be the same regardless of the method used to break up the bacterial cells.

A renewed investigation of bacteria by these methods may yield further information on their labile antigenic components, which may have been overlooked previously because of their inactivation by the methods used in extraction.

M41. On the Mutual Multivalence of Toxin and Antitoxin. HARRY EAGLE, Johns Hopkins Hospital, Baltimore, Maryland.

Diphtheric toxin and antitoxin do not necessarily combine in a fixed proportion to form a "neutral" compound, as postulated by Ehrlich and by Arrhenius and Madsen. Conforming to other antigen-antibody systems, both reagents seem multivalent with respect to each other. In the presence of excess toxin, antitoxin binds several times as much toxin as is indicated by its neutralizing activity, and the resulting compound is itself toxic. Similarly, in the presence of excess antitoxin, toxin combines with more than the neutralizing quantity of antitoxin to form an antitoxic compound. The precisely neutral compound, instead of being the usual type of combination, is formed only when the two reagents are mixed in just the proper proportions. The present experiments offer no clue as to the stoichiometric ratio between toxin and antitoxin at this point of equivalence (neutrality); nor do they indicate the number of reacting groups which bind each molecule of toxin and antitoxin at the equivalence point.

M42. The Quantitative Estimation of Staphylococcal and Other Bacterial Polysaccharides by Means of Leuco Crystal Violet Reagent. GEORGE H. CHAPMAN AND MERRITT H. STILES, Clinical Research Laboratory, New York, N. Y.

The leuco crystal violet reagent was the result of a refinement of the reaction between staphylococcal polysaccharides and leuco bases of the triphenylmethane dyes. It was prepared by dissolving 0.757 gram of 4,4′4″-hexamethyltriaminotriphenylmethane (Eastman Kodak Company No. 3651) in water by the aid of 6.44 cc. of N/1 HCl, and diluting to 100.0 cc. with water. Each cubic centimeter of reagent is equivalent

to 10 mgm. of white polysaccharide from *Staphylococcus aureus*. The reagent is standardized with neopeptone solution. An appropriate volume of the clear, peptone-free polysaccharide solution is measured into the bottom of a 75 x 11 mm. tube. The leuco crystal violet reagent is added from a micro burette with a micro tip until the initial turbidity completely redissolves. The amount of reagent required to reach this endpoint is proportionate to the amount of polysaccharide present. Other bacterial polysaccharides can be determined by this method. A statistical study is presented illustrating the precision of the method.

M43. Methods of Tracing Vi-antigens and Vi-antibodies. LASZLO DETRE, National Institute of Health, Washington, D. C.

A. *Vi-antigens:* (1) *Examination by comparative agglutination of cultures grown at 37° and at room temperature (23°C.) with a pure somatic serum.* (2) *Examination by absorption test.* One large loopful of a 24-hour 37°C. agar culture is suspended in a 1:100 dilution of a potent Vi antiserum (titer = 1:1600); the mixture is shaken every 30 minutes. After 3 to 4 hours, titration of the centrifuged supernatant is made against a purified and deflocculated Vi-type typhosus strain (see below). The titer differences indicate the approximate Vi content of the strain. (3) *Examination by the rabbit test.* Rabbits are treated intravenously with the live typhosus cultures to be examined. Proper amounts are $\frac{1}{8}$, $\frac{1}{6}$, $\frac{1}{4}$ loops; intervals, 3 days. The serum is drawn 6 days after the last injection and titrated against the mentioned test-emulsion (= deflocculated suspension of a purified Vi strain).

All three methods gave uniform results, showing that with the exception of the well known strain 0901 none of the examined typhoid strains was free from Vi antigens. It should be emphasized that strain H901 contains the Vi antigens. In using this strain as an antigen, serums of 1:40 – 1:80 Vi titer were obtained.

B. *Vi-antibodies.* The Felix method consists of exhausting the H and O content of the serum under study by the addition of sufficiently large amounts of H901 cultures and titrating it against Vi cultures (e.g., the Watson strain). This method seems to be inexact, therefore, another method is proposed as follows:

First step: Purification of the Watson or similar Vi-type smooth typhosus strain. (1) The O agglutination titer of the supposedly inagglutinable (Vi type) strain, grown at 37° is determined. (2) A double amount of the used O serum is added to the examined suspension. (3) After 20 hours (cool room) a small quantity of the perfectly clear supernatant is

removed and cultivated on agar. (4) This procedure is repeated 2–3 times. By this "selective agglutination" colonies will be obtained with greatly increased O resistance.

Second Step: Deflocculation of the "purified" Vi culture. One loop of a fresh 37° agar culture suspended in one cubic centimeter of saline is shaken for five minutes, then centrifuged for five minutes (2400 r.p.m.). After removal of the first supernatant fluid this procedure is repeated 4–6 times, until the supernatant fluid does not give the ink-test. About 50 agglutinin units of a powerful HO serum are added to 0.50 of the supernatant fluids and one drop of a one per cent solution of Higgin's India Ink in saline is added. The first three supernatant fluids will give an ink reaction; the fourth or fifth supernatant fluids will be negative. The suspension is now ready for use as a test liquid. By the purification of the strain, its O resistance is increased to such a degree that it can be regarded as fully O resistant; by the deflocculation it has lost all H components; it reacts, therefore, only with the Vi-antigens.

The suspension can be kept for about one week in the cool room. The addition of 0.1 per cent of formalin does not change its properties for about two weeks. It loses its O resistance within 48 hours after an addition of phenol.

The use of the described test-suspension for detection of even small amounts (1:5, for instance) of Vi agglutinins is advocated.

M44. Reactions of Pneumococcal Hemolysin with Certain Sterols. BARNETT COHEN AND HARRY SHWACHMAN, The Johns Hopkins School of Medicine.

1. The reactions with sterols were examined to throw light on the chemistry of the lysin. The digitonin-precipitable compounds: cholesterol, allocholesterol and coprosterol inhibited one hemolytic unit in doses of 0.00001, 0.00001 and 0.01 mgm., respectively. The digitonin-negative compounds: cholesterone and pseudocholestene, did not inhibit until 0.1 mgm. doses were reached; cholesteryl acetate failed to inhibit with 0.033 mgm., the highest concentration obtainable.

Reversibly inactive lysin (i.e., air-oxidized and capable of reactivation with cysteine or H_2S) is apparently unaffected by cholesterol, for the latent activity can be restored completely by reduction. On the other hand, active hemolysin after similar treatment shows no restoration, except occasionally to a slight degree which doubtless is due to small amounts of admixed reversibly inactive lysin. Known mixtures of the two forms act in agreement with this view. Apparently, therefore, the

presence and stereochemical configuration of the OH group in position 3 of the sterol molecule, which conditions the digitonin reaction, is also responsible for the interaction of the sterol with active lysin.

2. A concentrated active lysin solution (but not the reversibly inactive form) gives a positive nitroprusside reaction, indicating the presence of SH groups. When five to ten times the inhibiting dose of cholesterol is added, the SH-test remains positive. This indicates that the point of attachment for cholesterol on the lysin molecule is somewhere else than on the sulfhydril group which, according to other evidence, is associated with activity of the lysin on the red cell.

Although much free cholesterol is present in the red cell, we are not yet prepared to accept without more conclusive data the view that the red cell sterol attaches the lysin as an antecedent to hemolysis. Nevertheless, the following observations seem to indicate that such is the case. Active lysin is practically entirely removed by an equivalent amount of red cells. On the other hand, the reversibly inactive form is not, because it can be recovered in its entirety from the supernatant fluid of the red cells. Furthermore, red cells treated with cholesterol remain vulnerable to the action of active hemolysin.

It is expected that work in progress on the concentration and isolation of the lysin will yield material on which more definite conclusions can be obtained.

M45. The Acquisition of Heterophile Antigen by Living Bacteria. D. FRANK HOLTMAN AND GRANT L. STAHLY, Department of Bacteriology, Ohio State University, Columbus.

A series of experiments was devised to investigate Buchbinder's hypothesis that the antigenic properties of bacteria might be altered by their particular animal hosts. The object of these experiments was to determine whether bacteria originally lacking heterophile antigen could acquire such an agent from cultivation in horse serum media or in the tissues of guinea pigs, and whether the antigen thus acquired could be retained by the cells during subsequent propagation in synthetic media. *Eberthella typhi* and *Salmonella paratyphi* served as the experimental species. Cultures propagated by daily transfer on horse serum agar for a period of three weeks incited development of anti-sheep hemolysin in rabbits. Living cultures placed in collodion sacs and incubated in the peritoneal cavities of guinea pigs for 17 days, also exhibited the property of engendering anti-sheep hemolysin in rabbits. Killed cultures placed under identical conditions failed to exhibit heterophile phenom-

ena. All cultures used for rabbit inoculations were washed thoroughly in isotonic saline before injection. Cultures acquiring heterophile antigen retained some of the agent during daily transfer in synthetic media for three weeks, but had lost it after 11 weeks.

M46. Relationship of the Length of the Incubation Period to the Development of Hypersensitiveness. NORMAN J. MILLER AND PAUL S. PRICKETT, Bacteriological Laboratory, Mead Johnson and Co., Evansville, Indiana.

According to the literature, an incubation period of three weeks is considered ample for the development of hypersensitiveness in guinea pigs. However, at least with one class of compounds, namely dehydrated bananas, this period has been found insufficient, hence a study was made of the relationship of the length of the incubation period to the development of hypersensitivity.

Guinea pigs were sensitized by intraperitoneal injections on three consecutive days with extracts of dehydrated bananas; following this the animals were divided into three groups. After a three week incubation period one group received intravenous shock injections, while the second group was incubated for five weeks and the remaining group for seven weeks before they received intravenous shock injections. An adequate number of control animals was used with each group. All extracts were freshly prepared for each injection and were clarified and sterilized by filtration. After a three week incubation the animals showed no reactions to the shock injection, but those incubated five weeks exhibited definitely positive reactions and those incubated seven weeks all gave violent and fatal reactions. A repetition of this work using a different banana powder gave confirmation that an incubation period of seven weeks was superior to one of three or five weeks for determining the anaphylactogenic property of this product.

It is concluded that incubation periods as long as seven weeks are required for the determination of the anaphylactogenesis of some types of products.

M47. On the Prolonged Coagulation Time Subsequent to Anaphylactic Shock. HARRY EAGLE, C. G. JOHNSTON, AND I. S. RAVDIN, Johns Hopkins Hospital, Baltimore, Maryland.

The retarded coagulation observed in rabbits and dogs immediately after anaphylactic shock is regularly associated with the presence of increased amounts of antithrombin in the blood. The increased anti-

thrombic activity may be as much as 100 to 200 times the normal level. The fibrinogen content of the plasma is unaffected; and for the reasons cited in the text, there is reason to believe that even the plasmas completely non-coagulable by calcium and tissue extract nevertheless contain their normal quota of prothrombin and of platelets.

The increased antithrombic activity of the blood after anaphylactic shock is an adequate explanation of the observed retardation of coagulation.

M48. Further Evidence that Serologic Aggregation is Non-Specific. SANFORD B. HOOKER AND WILLIAM C. BOYD. Evans Memorial, Boston.

In mixtures of fowl and human erythrocytes and their mixed corresponding antisera, which did not cross-react, we regularly observed the formation of clumps which contained both kinds of cells simultaneously. This indicates that the second or aggregative stage of serologic reactions is non-specific and supports the observations of Abramson but not those of Topley, Wilson, and Duncan.

M49. Further Experiments on Whooping Cough. JOHN A. TOOMEY, Department of Pediatrics, Western Reserve University and Division of Contagious Diseases, City Hospital, Cleveland, Ohio.

Freshly isolated or phase I *Hemophilus pertussis* organisms are virulent for guinea pigs. They produce serums of high agglutinin titer in rabbits, but, nevertheless, cause nothing more serious in the human being than a rhinitis which is unassociated with fever or any subjective symptom of consequence. As the disease progresses the patient gradually becomes sensitized to the organisms and their endotoxins, and a lymphocytosis appears. The patient then begins to whoop and the most severe stage of the disease follows. Curiously, as the patient's condition gets worse, phase I organisms are less easily isolated. It only confirms the obvious fact that the whooping cough is not due to organisms *per se*, but to an exudate produced during the metabolism of *Hemophilus pertussis*, an exudate which is produced after the organism has become acclimatized to the patient. This exudate has been produced *in vitro* and has all the sticky qualities of the material coughed out by patients. It would be logical then to try to actively protect individuals against the mucoid exudate produced during the metabolism of *Hemophilus pertussis.* It would be equally logical to treat

those patients ill with the disease by injecting the specific factor in order that active immunity might be established more quickly. It may likewise be logical to confer passive immunity on those individuals who have the disease by injecting a serum made by using the specific mucoid factor as the stimulating antigen.

M50. A Group of Cultures Resembling both Bacillus pertussis and Bacillus bronchisepticus but Identical with Neither. GRACE ELDERING AND PEARL KENDRICK, Michigan Department of Health Bureau of Laboratories, Western Michigan Division, Grand Rapids.

A group of nine cultures isolated by the cough plate method from six whooping cough patients has been described. The cultures were compared with *Bacillus pertussis* and *Bacillus bronchisepticus* with respect to morphology, growth on various media, biological characteristics, serological reactions including agglutinin absorption tests, and certain reactions in animals. These cultures show certain relationships with both *Bacillus pertussis* and *Bacillus bronchisepticus*, but vary from each in certain important points. Some theoretical and practical implications of such a group are pointed out and discussed.

M51. The Susceptibility of the Guinea Pig Fetus to a Strain of Actinomyces. FRED W. GALLAGHER AND ORAM C. WOOLPERT, Department of Bacteriology, Ohio State University, Columbus.

Since the guinea pig fetus had been shown by Woolpert and associates to be relatively susceptible to infectious agents, it was hoped that it might prove reactive to certain members of the genus *Actinomyces* for which no suitable experimental animal has been available. To test this hypothesis, an American Type Culture Collection strain, designated *Actinomyces bovis* Harz, and a smooth variant derived therefrom, were inoculated into fetal guinea pigs, newborn and adult guinea pigs, fetal and adult rabbits, and adult white mice.

The procedure of fetal inoculation involved surgical exposure of the maternal uterus and needle puncture through the uterine wall into the fetal brain. The fetuses were later delivered by cesarean section and examined grossly and bacteriologically. Post-natal animals were inoculated intracerebrally or intraperitoneally.

The original rough strain produced no visible effects in any of the animals nor could it be recovered in culture from their tissues 24 hours or more after inoculation. The smooth variant, however, killed fetal

guinea pigs within 24 hours, but failed to affect the other animals tested. The variant retained its virulence through several fetus-culture-fetus passages. In two instances other types of variation occurred during fetal passage. Heat-killed controls of the variant were without effect.

It is concluded that the fetal guinea pig is a suitable experimental animal for the demonstration of pathogenicity of members of the *Actinomyces* group, and further, that smooth variants of *Actinomyces* are more likely to be virulent than those in the rough phase.

M52. Experimental Lobar Pneumonia in the Rat; A Method for the Study of Therapy in this Disease. W. J. NUNGESTER, Hygienic Laboratory, University of Michigan.

A method for producing lobar pneumonia in the white rat has been previously described. This disease has been studied in some 2,000 rats, employing strains of type I, II and III of the pneumococcus. The conditions met with in these experimental lesions, which closely resembled those seen in lobar pneumonia in man, are definitely different from those encountered in pneumococcus peritonitis or septicemia produced in laboratory animals. Hence it seemed that the use of this method for producing lobar pneumonia in the rat might have certain advantages in studies directed at the problems of treatment of this disease. It was the purpose of this work to determine whether or not the course of experimental pneumonia in the rat could be altered by therapy.

Various therapeutic procedures were employed to treat rats in which pneumonia had been induced. These procedures included the use of atropine sulphate, pilocarpine hydrochloride, morphine sulphate, increased oxygen tension and a commercial pneumococcus antiserum. The latter had the most marked effect. The results noted were a lowering of the percentage of mortality and the incidence of pleurisy, a slight lessening of the effect of the disease on body temperature, and a decrease in the bacteremia. These results were obtained with type I and II infections. Type III infections were not influenced by serum therapy.

M53. Observations on the Effect of Prontosil and a Related Compound in Hemolytic Streptococcus Infections. PAUL GROSS, FRANK B. COOPER AND RALPH R. MELLON, The Western Pennsylvania Hospital, Institute of Pathology.

The interesting communications of Colebrook and Kenny and of Buttle, and other quoted authors on the protective and curative effect in

mice of the new chemical compound "prontosil," disclose a remarkable paradox. That is to say, mice infected with highly virulent mouse-passage strains of human origin showed a much higher percentage of recovery than mice infected with non-passage human strains of low virulence. Realizing the effect of strain diversity in mice, repetition with other strains seemed desirable.

Accordingly, the Pion strain (Pasteur Institute) of relatively low virulence, and our own strain (Stoddard, human type) of high virulence were employed. Although definitely favorable effects are to be noted with the Stoddard strain, no comparison can as yet be drawn between it and the Pion, owing to an unexpected fluctuation in virulence that has occurred in the Stoddard. But with the Pion strain only one out of ten controls was alive after 15 days, while six of the treated mice were alive and apparently normal. Similar results were obtained with the already known para-aminobenzenesulphonamide compound synthesized by one of us (Cooper). Several hundred mice are being studied.

Several clinical cases appeared to be favorably affected, but only one—a case of meningitis due to a hemolytic streptococcus—was of high evidential value. No final conclusions can be drawn.

We are much indebted to the Winthrop Chemical Company for our supply of prontosil.

M54. The Types of Hemolytic Streptococci in Grammar School Children. John Hays Bailey, Chicago Branch Laboratory, Illinois Department of Public Health, Chicago.

This report is concerned with the types of *Streptococcus hemolyticus* found in the noses and throats of the grammar school children of two small towns and of children in another grammar school where an epidemic of scarlet fever was occurring.

In each case the types of hemolytic streptococci most frequently encountered were 1, 2, 3, 4, and 6 (Griffith's classification). In the school where an epidemic of scarlet fever was occurring, types other than 1, 2, 3, 4, and 6 (the types most frequently encountered in clinical cases of scarlet fever) were encountered in only six instances, while in the other schools types other than these five occurred approximately five times as frequently.

The toxin production of 183 cultures of the hemolytic streptococci was tested and 161, or 88 per cent, proved toxic.

M55. The Occurrence of Diplococcus pneumoniae in Urinary Tract Infections. H. D. Moor and Ida Lucille Brown, Depart-

ment of Bacteriology, University of Oklahoma School of Medicine.

Because of the frequent occurrence of Gram-positive diplococci in urinary specimens brought in for examination, the problem was undertaken of identifying this organism and establishing its etiological relationship to urinary tract infections.

Three hundred and nineteen urine specimens were cultured on brain-heart infusion broth. Two hundred and sixty-four were from patients suffering from urinary trouble, the remainder from apparently healthy medical students. From 79 of the former cases a pure culture of a Gram-positive lancet-shaped diplococcus was obtained. Each culture was transferred to 25 different media, including serum-inulin water, and each tested for solubility in bile. Pathogenicity was tested by inoculation of white mice and serum reactions were used for further identification. An autogenous vaccine was prepared from each pure culture isolated.

The organism was found to be morphologically, culturally and serologically identical with the pneumococci and agglutinated specifically with Type XIV antipneumococcic serum. Administration of autogenous vaccine has given encouraging results to date. In three cases the organism was obtained from the blood stream and at the same time isolated from the urine. In four cases the organism was found in dental infections with the urinary tract also involved. After the offending teeth were removed the organism could no longer be isolated from the urine and symptoms disappeared. Such an organism could not be isolated from the urine of any of the 55 controls.

M56. The Survival of Eberthella typhosa in Soil. PAUL J. BEARD, J. M. CARLSON AND R. D. CHAMBERS, Stanford University.

An investigation has been made of the survival time and rates of death of *Eberthella typhosa* in various types of soil when exposed to the natural variations of temperature and moisture under *out of door* conditions. The various soils in unglazed clay pots were inoculated and placed out of doors in such a position that they were exposed to rain at any time and to the sun about two-thirds of the day. The soils were initially adjusted to approximately the same moisture content and moisture determinations were made with the bacterial counts.

At intervals, portions of the soil were suspended in saline, plated and the numbers of survivors per gram of soil were determined. The Difco modification of Wilson and Blair's bismuth sulfite medium was utilized

for the determination since experiments indicated that practically all the normal soil types were inhibited without any significant inhibition of the typhoid bacilli.

The results demonstrated, as was to be expected, relationships between the moisture-retaining power of the soil, the temperature and rainfall, and the rates of death and total survival time of the implanted organisms.

The maximum survival period of *Eberthella typhosa* as shown by the series was six weeks in an adobe clay mixture during the rainy season. During the dry season survival in the same soil was three weeks.

M57. Succession of Colon-Typhoid Organisms in Normal Human Feces. LELAND W. PARR, The George Washington University.

In a study of the normal flora more than 6000 strains obtained from direct plating of 210 fecal specimens from 32 healthy babies and 59 normal adults were investigated, over 1700 in pure culture detail, the balance only for the utilization of citrate as a sole carbon source. Ample direct plating on Endo and on citrate agar failed in 16 specimens. From 11, including one formula baby and four adults, even enrichment of the specimen did not yield group organisms.

Colon bacilli were encountered in 92.3 per cent, aerogenes in 25.2 per cent and coli-aerogenes intermediates in 20 per cent of the specimens. Paracoli were found 21 times, cellobiose-fermenting coli 52 times, H_2S-positive coli once, gelatin-liquefying coli once, chromogenic coli twice, *Bacillus coli-mutabile* 8 times, *Eberthella typhosa* twice, gelatin liquefying aerogenes 14 times and *Pseudomonas* twice. *Shigella, Salmonella, Proteus* and *Alcaligenes* were not found. Bacillus "P" of Clemesha(?) was isolated from 11. This is the typical group of bacteria found in long stored feces and is often confused with pathogenic group members.

One subject was examined 92 times and of others multiple examinations were made. Serial study adds to the evidence from single studies that the fecal flora of the healthy is subject to such great variation that a number of points in sanitary, systematic and pathologic bacteriology may have to be restated.

M58. The Relationship of Bacillus siamensis and Similar Pathogenic Spore-Forming Bacteria to Bacillus cereus. FRANCIS E. CLARK, University of Colorado School of Medicine and Hospitals, and United States Department of Agriculture, Washington, D. C.

Pathogenic, motile, aerobic sporulating bacilli isolated from a contaminated blood culture and from air and soil were tentatively identified as *Bacillus siamensis* (Siribaed). A comparative study of this *Bacillus siamensis* group with type cultures of aerobic spore-formers such as *Bacillus cereus*, *Bacillus subtilis*, *Bacillus megatherium* and others showed *Bacillus siamensis* to be culturally indistinguishable from *Bacillus cereus*. Although the *Bacillus siamensis* cultures were pathogenic when first isolated, loss of pathogenicity occurred until their virulence was comparable to that exhibited by *Bacillus cereus* after rapid subculture of this species on blood agar. It is, therefore, concluded that *Bacillus siamensis* is identical with *Bacillus cereus*. Since *Bacillus cereus* is one of the most common of the aerobic spore-formers in soil, it is probable that many of the "motile, anthrax-like" and "pathogenic subtilis" bacilli isolated by earlier workers were likewise *Bacillus cereus*.

AGRICULTURAL AND INDUSTRIAL BACTERIOLOGY

A1. The Anaerobic Bacteria of the Soil—a Tillable Field. IVAN C. HALL, University of Colorado School of Medicine and Hospitals, Denver.

Present knowledge of the anaerobic bacteria of the soil has been developed along two distinct lines by workers interested in the soil from widely differing viewpoints. On one hand soil bacteriologists undertook to solve the complicated problems of decay in the soil, fermentation, putrefaction, nitrogen fixation, oxidation, reduction, and general microbic ecology in the interest of soil fertility, while on the other, medical bacteriologists studied the anaerobic flora of the soil in relation to malignant oedema, gaseous gangrene, and tetanus caused by wound infections and in relation to botulism caused by eating improperly processed foods contaminated with soil.

The early observations of Mitscherlich (1850) on decay of potatoes, the discovery of *Amylobacter* by Trecul (1865), and van Tieghem's (1877–1879) studies on the decomposition of cellulose laid the background for the later work of Beijerink (1896–1902) on *Granulobacter*, and of Winogradsky (1893–), Omeliansky (1895–1916), and others on *Clostridium pastorianium*. There is still much confusion as to the exact taxonomic position of the anaerobic bacilli concerned in the decomposition of cellulose in the soil and in the fixation of nitrogen; new studies are needed to redefine the important species involved in these processes.

The taxonomic status of the anaerobic bacilli of the soil concerned

in the production of disease seems to be much better established, but much remains to be learned as to their distribution in many parts of the world and in all kinds of soil environments, with special reference to the application of the fecal and telluric theories of origin.

Beginning with Koch's experimental production of malignant oedema by inoculation of soil suspensions in 1876 it is possible to list the isolation of many pathogenic and non-pathogenic anaerobes from the soil. But most of these records were incidental to other studies and there is a definite opportunity now for further comprehensive surveys similar to those reported by Meyer and his associates in reference to *Bacillus botulinus*, by Zeissler on the anaerobic flora of the German war fronts, and by Sasaki in Japan.

The bacteriological analysis of soil samples presents most difficult technical problems owing to the large number of variable chemical factors and the almost constant presence of aerobic sporulating bacilli which greatly complicate the isolation of the anaerobes. But the present state of bacteriological art and science is such as to encourage new attacks upon these problems.

A2. Evidences of Localization of Microörganisms about Plant Roots. ROBERT L. STARKEY, Department of Soil Microbiology, Agricultural Experiment Station, New Brunswick, N. J.

The Rossi-Cholodny contact slide method was used to obtain information concerning the influences of root systems of plants upon the microorganisms in the soil. Microscope slides (50 x 75 mm.) were buried vertically in the soil. Seeds or seedlings were planted about one to three inches above the slides. During plant development, roots passed over the surfaces of the slides and adhered to them. Organisms growing about the roots and soil particles also became fixed to the slides and could be distinguished after being stained. The limited number of observations so far made demonstrate that various microörganisms develop about plant roots in greater numbers than in the soil mass free from root growth. Bacteria and actinomycetes were found in masses in contact with the roots; smaller aggregates and isolated cells were found elsewhere on the slides. Short rods and coccoid bacterial cells predominated; thin rods and *Azotobacter*-like cells were occasionally encountered.

A3. Fungous Mycelia in the Soil. CHARLES THOM AND MARIE BETZNER MORROW, Bureau of Plant Industry, Washington, D. C.

Soil fungi may possibly be divided into two sections: (1) Those capable of living normally in relation to soil organic matter in the chemical sense, i.e., to residual products of decomposition; and (2) those concerned in primary decomposition, i.e., the breakdown of plant and animal remains toward those residual products.

The distinction is mainly on paper. Certain mushrooms, and such organisms as *Actinomyces, Penicillium luteum, Zygorrhynchus* and possibly *Trichoderma,* appear to be quite able to cause *in vitro* the decomposition of the residual products regarded by the chemist as "humus." They probably account for part, at least, of the slow but continuous evolution of carbon dioxide observed from soil otherwise practically static, but they also grow beautifully in culture as causes of primary decomposition if given the chance.

The majority of soil fungi are directly related to the general disintegration of plant remains in and on the surface of the soil. The relation of a root-rot organism to the host root system is therefore sketched as an example of the relationship of soil fungi to organic remains.

A4. Respiratory Enzyme Systems in the Root Nodule Bacteria. P. W. WILSON, Biochemical Laboratory, University of Cambridge and Department of Agricultural Bacteriology, University of Wisconsin.

Development of methods for use of non-proliferating cells, i.e., the so-called "resting cells," constitutes a notable advance in technique for study of bacterial enzyme systems, especially those concerned with respiration. Mass suspensions from agar or liquid cultures are washed free from nutrients and resuspended in phosphate buffer solutions, and hydrogen transfers from various substrates are followed in micro-respirometers, Thunberg tubes or by micro-chemical determinations. Free oxygen, dyes (methylene blue), inorganic compounds, as KNO_3, etc., may be employed as hydrogen acceptors. Among the advantages of the technique are: (1) Respiratory activities are separated from those associated with growth. (2) Kinetics of reactions may be determined which allows comparison of rates of reactions instead of total effects. (3) Single reactions may often be studied rather than the complex of reactions occurring with growing cells. (4) Employment of specific inhibitors allows some separation of related reactions. (5) Results are referred to a comparable basis (as QO_2) eliminating effects which arise from differences in total or rate of growth. (6) Experiments are short-time (1 to 2 hours) which eliminates, in part, differences in metab-

olism associated with cells in various stages of development and allows comparison of these differences.

These cited advantages will be illustrated by a consideration of some of the researches of the Cambridge University school, especially those concerned with the hydrogen enzymes of *Escherichia coli* and with the "Stickland reaction."

Before detailed studies of the various respiratory enzyme systems in a given species may be undertaken, examination of the chief respiration functions of the organism is necessary in order to determine the factors which influence the reactions. Unless these characteristics are defined, results may be misinterpreted, since they will represent a complex of competing influences rather than the single reaction under consideration. The type of preliminary investigations necessary will be illustrated by discussion of studies made with *Rhizobium trifolii.* When this organism is grown on the laboratory media usually employed, the bacteria are low in nitrogen, high in gum, and have a low rate of oxygen consumption (QO_2 on glucose, from 1 to 5). Moreover the endogenous respiration, i.e., respiration in absence of added substrate, is 50 to 70 per cent of that in the presence of glucose. If the organisms are grown on an agar substrate containing yeast extract plus mineral salts but *no carbohydrate*, the cells are practically free from gum, contain 10 per cent nitrogen, and possess relatively high respiratory activity (QO_2 on glucose, 50); also the endogenous respiration of these cells is only 10 to 20 per cent of that in the presence of glucose.

The influence of the following factors on the respiration of *Rhizobium trifolii* grown on this yeast extract medium was investigated: (1) Temperature, (2) pH, (3) concentration of phosphate in buffer solution, (4) concentration of substrate, (5) age of culture, (6) composition of medium with respect to yeast extract and carbohydrate, (7) concentration of suspension of organisms, and (8) stability of dehydrogenases. In addition, the rate of oxygen uptake on 21 substrates, including carbohydrates, polyhydric alcohols and salts of organic acids, was determined and compared with activation of these substrates using methylene blue as the hydrogen acceptor. The results of these studies will be presented and briefly discussed.

A5. Studies in the Mechanism of Symbiotic Nitrogen Fixation: Hydrogen as a Specific Inhibitor. W. W. UMBREIT AND P. W. WILSON, University of Wisconsin.

Physical-chemical studies on the pN_2 function of symbiotic nitrogen fixation supply evidences that commercial hydrogen may act as a

specific inhibitor for the fixation reaction in red clover inoculated with efficient strains of *Rhizobium trifolii*. Establishment of this finding is extremely important for elucidation of the mechanism of the process, since not only does it throw light on the nature of possible enzyme reactions involved but also provides a convenient tool for future research on this problem. Because of this importance, numerous researches have been carried on for the past three years directed toward the answer of the two crucial questions:

1. Is the inhibition noted in the development of plants grown in atmospheres containing hydrogen specific for the fixation reaction, or is it on the general growth of the plant?
2. Does the inhibition arise from the hydrogen itself or from an impurity in the gas used?

Data concerned with the first question have been discussed previously (Wilson: Trans. Second Microbiol. Cong., London, 1936); these support the view that the inhibition is specific for the fixation process. Experiments reported in this paper deal with: (1) attempts to locate the effective impurity in commercial sources of hydrogen; (2) effect of hydrogen from different sources. All results to date indicate that the observed inhibition is an effect of hydrogen as such and not due to an accompanying impurity.

A6. Adsorbed Calcium on Colloidal Clay and an Accessory Growth Factor in Laboratory Production of Rhizobium Cultures. WM. A. ALBRECHT AND THOS. M. MCCALLA, University of Missouri.

Calcium adsorbed on colloidal clay which also carried other nutrient cations, was found responsible for transforming abnormal forms of *Rhizobium* cultures to the normal, and for improving their inoculating ability. Substitution of barium for calcium on the clay transformed the normal colonies to the abnormal yellow, orange or red forms with low inoculating power. The colloidal clay medium grew the organisms at acidities as high as pH 5.0 and delivered calcium more effectively than the agar medium with a calcium carbonate suspension. These results point to the need for generous amounts of calcium in the nutrition of *Rhizobium* to make it an effective inoculator as well as in the legume plant to make the symbiotic relationship between these two most effective for nodulation and nitrogen fixation.

A search for an accessory growth factor more effective than yeast in laboratory cultures led to that present in kraut juice, a part of the stimulative effect of which is the result of the available nitrogen content.

Studies on the nature of the responsible factor show it soluble in ethyl alcohol, dilute acetic acid, and water, but slightly soluble in methyl alcohol and insoluble in petroleum ether and pyridine. It does not seem to be adsorbed on Fuller's earth. It passes through the collodion bag during dialysis, but is inactivated or destroyed on electrodialysis for complete chloride removal. Partial electrodialysis at low voltage to a clear solution and parchment dialysis are not detrimental.

The behavior of the growth factor suggests a molecule of colloidal dimensions but of small magnitude and an activity associated with the oxygen consumption by the organism. A colloidal clay suspension with kraut juice and sugar additions was found the most effective medium for rapid multiplication of effective *Rhizobium* cultures. The response by the *Rhizobium* as measured by turbidity developments related to quantities of the growth factor suggests the consideration of microörganisms as rapid aids in the assay of substances for their contents of such factors.

A7. Attempts to Differentiate the Species of Rhizobium by Physiological Methods. O. A. Bushnell and W. B. Sarles, Department of Agricultural Bacteriology, University of Wisconsin, Madison.

In order to determine whether or not there exist any demonstrable physiological differences among the several species of root-nodule bacteria, representative strains of each of the common *Rhizobium* species were tested for their ability to grow and to bring about measurable changes in a number of different culture media in which the sources of carbon or of fixed nitrogen were varied.

The sugars arabinose, cellobiose, galactose, sucrose, and xylose were used as carbon sources in a liquid medium in which sodium nitrate was the nitrogen source; urea and nine different ammonium salts of inorganic and organic acids: ammonium nitrate, chloride, sulfate, phosphate, acetate, citrate, lactate, oxalate, and tartrate, were employed as nitrogen sources in a liquid medium in which mannitol was the source of carbon.

In all of these tests changes in the reaction of the culture media were too variable to be dependable: variations among the strains of one species were often as great as were the differences that were noted between species. In general, however, two large physiological groups of rhizobia were differentiated by both the sugars and the ammonium salts: the one, characterized by the formation of an acid reaction in these media, was made up of the alfalfa, clover, pea, bean, and *Dalea* strains; the

other, characterized by the formation of an alkaline reaction, included most of the strains from the soybean-cowpea-lupine group. Other than this two-fold separation there were found, in these tests, no physiological characteristics which could be used to distinguish any one species of *Rhizobium* from the others.

A8. Nitrogen Transformations in Certain Colorado Soils. HERBERT W. REUSZER, Colorado Agricultural Experiment Station, Fort Collins.

In an effort to determine whether appreciable increases in nitrogen content could be brought about as a result of non-symbiotic biological fixation, 12 Colorado soils were incubated in the laboratory for a period of 120 days. The soils were kept at a temperature of 28°C. and a moisture content of 20 per cent. Two pots of each soil were incubated untreated and two with one per cent cellulose (ground filter paper) added. At intervals of 30 days duplicate samples from each pot were analyzed for total nitrogen by the Kjeldahl-Gunning method, modified to include nitrate nitrogen. The latter form of nitrogen was also determined separately.

Rather large quantities of nitrate accumulated in all soils to which no cellulose had been added. It would seem that in most cases this accumulation was sufficiently large to indicate that the nitrogen-fixing organisms secure their nitrogen from the soil rather than from the atmosphere. Few of the variations detected in total nitrogen content of the soils were found to be statistically significant. There was no consistent increase in total nitrogen with increasing length of incubation. No consistent differences could be detected in nitrogen content between soils with and those without added cellulose.

The data were treated statistically by the analysis of variance method of Fisher. In the final averages for nitrogen content of soils with and without cellulose, where 128 Kjeldahl analyses entered into each average, a difference of 0.0015 per cent nitrogen was required for significance. In the averages for each of four periods, where 64 analyses were included, a difference of 0.0021 per cent was required. Where 32 analyses entered into the averages for each of eight soils, a difference of 0.003 per cent nitrogen was required for significance. Even with the large number of analyses entering into these results, the differences required for significance were larger than those frequently taken as indicating nitrogen fixation for smaller numbers of samples.

A9. Some Factors Affecting the Preparation and Use of Silica-Gel Media for the Growth of Non-Symbiotic Nitrogen-Fixing Bacteria. HAROLD W. BATCHELOR, Ohio Agricultural Experiment Station, Wooster.

A preliminary survey of the effects of fertility practices on the number of naturally occurring *Azotobacter* and *Clostridium butyricum* colonies in plot soils has been previously reported. Preliminary reports have been made on the effects of rates and lengths of time of shaking a soil suspension and of its settling on the apparent populations of *Azotobacter* in soil.

A medium more satisfactory for the growth of *Azotobacter* than the one previously used has been developed together with a satisfactory medium for enumerating the populations of *Clostridium butyricum* in soil. The effects of sodium and potassium silicates singly and in mixtures, of different sources of nitrogen in different concentrations, of the anions ($-SO_4$), ($-Cl$), and ($-PO_4$) singly and in mixtures, of different sources of organic matter for energy, and of the quantity of medium used in the petri dishes have been studied. Electrometric titrations of representative silicate and acid mixtures which show the limitations of the use of the media, and the compositions of the media finally adopted are reported.

A10. The Purification of Sewage by Bacteria in Pure Culture. C. T. BUTTERFIELD, U. S. Public Health Service, Stream Pollution Investigations, Cincinnati, Ohio.

In investigating the activated sludge process of sewage treatment, an entirely biological process, the oxidation, adsorption and synthesis phases of purification are being considered in a combined study by the laboratory staff. The present report deals with the oxidation of sewage by massed growths of pure cultures of bacteria. Predominant bacteria from good activated sludge were isolated in pure culture. Growing these pure cultures in sterilized natural sewage and in sterile synthetic sewage, massed cultures (pure culture "activated sludges") were developed by the fill and draw method of feeding. Dividing each of these pure massed cultures into two aliquot portions (one for a control and one for testing its oxidative properties on a substrate feed, either sterilized natural sewage or sterile synthetic sewage), the oxidizing efficiency of the massed cultures under aeration was determined. At the same time, the 5-day biochemical oxygen demand of the substrate feeds which varied from 142 to 345 p.p.m., was determined by the usual excess-

oxygen method. The results indicate that these massed cultures (pure culture "activated sludges") can oxidize in 5 hours from 38 to 62 per cent of the 5-day biochemical oxygen demand of the substrate feeds.

A11. The Effect of Trickling Filters on the Bacterial Count of Sewage. H. O. HALVORSON AND LOUIS J. BIERMAN, Department of Bacteriology and Immunology, University of Minnesota.

Our studies indicate that there is no correlation between the ability of a trickling filter to reduce the oxygen demand of sewage and its ability to reduce the bacterial count. In ordinary trickling filters, the bacterial count is generally reduced from 60 to 90 per cent. The reduction in count, however, is a function of the number of organisms present in the raw sewage. If the waste applied to the filter contains few organisms, there may be a larger number present in the effluent than in the influent. The bacterial flora of the effluent appears to be more or less constant and independent of the flora of the influent. To get a reduction in bacterial count, it is necessary that the sewage applied to the filter contain a large number of organisms. Since this is usually the case, trickling filters as a rule produce effluents with fewer organisms than are found in the influents. Slides placed in the effluent of a trickling filter showed a wide variety of organisms. There appears to be no correlation between the morphological characteristics of a flora and the efficiency of the filter.

A12. The Distribution of Heterotrophic Bacteria in the Bottom Deposits of Lakes. ELIZABETH MCCOY AND ARTHUR T. HENRICI, University of Wisconsin and University of Minnesota.

Fifty-six profile samples of bottom deposits have been collected from 13 lakes of glacial origin in Minnesota and Wisconsin. These lakes show a wide range in type and productivity. The bottom deposits included sand, marl, various types of black ooze (gyttja) and brown ooze (dy).

Three hundred and twenty-two plate counts of bacteria have been made from varying levels of these samples. From the mud-water surface these have ranged from 6000 to 500,000,000 per cubic centimeter (mean, 370,600). The plate counts show a marked decrease with depth in the mud. Counts from the 30 to 33 cm. levels ranged from 120 to 275,000 per cubic centimeter (mean 21,000). When the counts are plotted against depth, curves of individual samples show much

variation and irregularity, though there is a general tendency to drop rapidly in the first 10 to 12 cm., more slowly beyond. Statistical treatment of the entire series of counts indicates that a logarithmic curve will best fit the data. Such a curve is similar to a survivorship curve from a disinfection experiment, and is interpreted as indicating that bacterial activity at the bottoms of lakes is carried on almost exclusively at the mud-water surface, the bacteria dying below this level.

The means of the counts from the upper 18 cm. from profundal stations show a fair correspondence with the productivity of the lakes, being highest (609,300) in the very eutrophic Lake Mendota, and lowest (2160) in the very oligotrophic Crystal Lake. Counts from littoral stations are much higher than those from profundal ones if the shoreward zone is occupied by aquatic plants, lower in the case of a sandy beach.

A 13. Viability of Bacteria in Sea Water. SELMAN A. WAKSMAN AND MARGARET HOTCHKISS, Woods Hole Oceanographic Institution, New Jersey Agricultural Experiment Station and New York Medical College.

Certain factors are responsible for the low numbers of bacteria usually found in natural sea water. The addition of a culture of an agar-liquefying marine bacterium to fresh sea water resulted in its rapid destruction; the same culture added to water sterilized by heat or by filtration through a Berkefeld survived for a considerable period of time. The destruction of the bacteria in the fresh sea water was not accompanied by a reduction in the consumption of oxygen. The surviving bacteria in the sterile water consumed less oxygen during a certain period of incubation than the rapidly reduced numbers of bacteria in the fresh water. The agents responsible for the destruction of the bacteria were thus shown to be biological in nature. Protozoa and other marine animals belonging to the animal plankton were found extensively in the fresh water where reduction in bacterial numbers took place. These organisms are believed to be at least partly responsible for the reduction in the bacteria in the sea water. The limited numbers of bacteria under natural conditions in the sea can thus be explained by a state of equilibrium between bacterial multiplication and bacterial destruction by the animal members of the plankton. A change in this equilibrium can be brought about by a change in the food supply and environmental conditions, such as temperature and aeration.

A14. The Influence of Solid Surface Upon the Physiological Activities of Bacteria in Sea Water. CLAUDE E. ZOBELL, Scripps Institution of Oceanography, University of California, La Jolla.

During the storage of raw sea water which initially contains only a few bacteria per milliliter the bacterial population may increase to millions per milliliter. The densest populations appear in the smallest receptacles in which the water is stored. This is attributed to the beneficial effect of solid surface on bacterial activity. Increasing the solid surface with glass beads, glass wool, silica sand or inert colloids tends to increase the population up to a level where available nutrients become a limiting factor.

Oxygen consumption, denitrification, ammonification and the fermentation of soluble carbohydrates are also favored by solid surface although the rate of these activities is not proportional to the bacterial populations. There is little or no chemical evidence of such activities until the bacterial population is actually decreasing and the rate continues to accelerate after the *phase of readjustment* in the growth curve is reached. This indicates that there are many biochemically active bacteria which are not demonstrated by plating procedures. Direct microscopic observations by the Henrici technique reveal that there are more bacteria tenaciously attached to glass surfaces than are found in the water.

Solid surfaces are believed to enhance the physiological activities of bacteria in several ways: (1) They may concentrate the dilute nutrients and exo-enzymes by adsorption or otherwise. (2) The interstices at the tangent of the bacterial cell and the solid surface may serve as concentration foci which retard the diffusion of exo-enzymes and metabolites away from the cell thereby favoring both digestion and absorption of foodstuffs. (3) These interstices may aid the production of optimal oxidation-reduction or other physico-chemical conditions. (4) Many marine bacteria are known to be obligate periphytes which are active only on a solid surface.

A15. Conditions Controlling the Marine Bacterial Population and Its Activity in the Sea. CHARLES E. RENN, Woods Hole Oceanographic Institution and Harvard Biological Institution.

It is striking that the bacterial population of the sea is much lower than that of fresh waters, soils, fermenting juices, or of other sites bearing decomposing organic matter. When the limitations of conventional counting methods are compensated for, it still appears that

the bacterial flora of the sea, even that peculiarly marine, is a suppressed population. Whenever sea water is stored alterations take place that permit a level of activity comparable to that in other very dilute media. These storage experiments have indicated some of the factors responsible for this unexpectedly low level of bacterial life in the ocean.

Concentrations of essential nutrients are much lower in the sea than in media with which bacteriologists are familiar, and the demands of other forms for particulate organic matter further depresses this already low content. Aside from shallows, shelf fringes and a thin superficial layer, the sea's greater volume exists at temperatures near freezing to which the specialized marine bacteria are not uniquely adapted. It appears, too, that a predatory nannoplankton, favored by lower temperatures, is active in grazing off the bacteria.

Particulate substrates, necessary for favorable development of potentially large attached populations, tend to settle and carry large numbers of bacteria into the mud during sedimentation, where they may or may not be active.

Detailed consideration of chemical and hydrographic processes in the sea is necessary for evaluating the function of bacteria in the ocean's living economy, and it is necessary that laboratory experiments be scaled with appreciation of these processes.

A16. Direct Microscopic Evidence of an Indigenous Bacterial Flora in Great Salt Lake. W. WHITNEY SMITH AND CLAUDE E. ZOBELL, University of Utah and Scripps Institution of Oceanography.

When chemically clean, sterile glass slides are suspended in Great Salt Lake, water of which contains 336 grams of salt per liter, bacteria attach themselves thereto. After 12 hours many attached cells can be discerned and some appear as micro-colonies. The longer the slides are left in the lake the larger and more numerous become the colonies. Controls show that this does not occur with lake water inoculated with bacteria from other sources and that killed bacteria do not become attached to slides.

A17. A Comparison of MacConkey's Bile Salt Lactose Broth and Standard Lactose Broth as Presumptive Test Media for Use in Water Analysis. MICHAEL A. FARRELL, The Pennsylvania State College.

Raghavachari (1936) as a result of an extensive study of various American presumptive test media with MacConkey's broth on Indian

water supplies, found that MacConkey's medium was the most efficient presumptive test medium examined. In view of these results he recommends that the Standard Methods Committee include MacConkey's broth in the comparative study they are making of presumptive test media.

Earlier work by the author had indicated that MacConkey's medium was inferior to standard lactose broth. In view of Raghavachari's result this problem was further investigated. The relative efficiency of standard lactose broth, MacConkey's medium and MacConkey's medium as modified by Raghavachari was tested on 17 pure cultures of the coli-aerogenes group (using the method of Butterfield and Hoskins) and 94 surface and well water samples. The results obtained indicated that standard lactose broth was much more efficient in the detection of small numbers of pure cultures of the coli-aerogenes group than either of the MacConkey broths.

Standard lactose broth was also found to be more efficient than either of these two other presumptive test media in testing 94 water samples.

A18. The Bacteriostatic Action of Brilliant Green in Solid Media on Members of the Colon-Aerogenes Group and Their Intermediates. ARNOLD E. HOOK AND E. R. HITCHNER, Department of Bacteriology, University of Maine, Orono.

A 1:1000 aqueous solution of brilliant green was added to nutrient agar (Difco), pH 7.0, in sufficient amounts to give the following concentrations in thousands: 1:25, 1:33, 1:50, 1:100, 1:200, 1:330, 1:400, 1:660, and 1:833. Duplicate plates of each concentration were inoculated by streaking with a standard 2 mm. loopful of a 1:10, 1:100, and 1:1000 dilution of a 24 hour-old broth culture of the various cultures and the inhibitory effect was observed after varying periods of incubation at 37°C.

All cultures exhibited a greater dye tolerance when the larger inocula were used. The *Aerobacter* strains grew at a much higher dye concentration than the *Escherichia* strains. Within the genus the various cultures of both *Escherichia* and *Aerobacter* exhibited marked differences in dye tolerance. These differences were more marked in representatives of the *Escherichia* group. Upon prolonged incubation many cultures produced visible growth at a dye concentration which inhibited development during a shorter incubation period.

A preliminary study with a few representative strains of *Escherichia coli* and *Aerobacter aerogenes* showed that when a 1:10 dilution of a 24-hour-old culture of the various strains was streaked on nutrient agar

containing a 1:200,000 concentration of the dye, all *Escherichia* cultures showed no visible growth after 48 hours of incubation while all *Aerobacter* strains grew rapidly. Further tests with 103 cultures, comprising 19 *Aerobacter* strains, 61 *Escherichia* strains, and 23 intermediates, differentiated by the M. R., V. P., indol and the citrate and cellobiose utilization tests, confirmed the results from the preliminary study with the *Escherichia* and *Aerobacter* strains. The results with the intermediates were irregular, since there was no definite relationship between any specific test and the dye tolerance.

The authors suggest the above procedure as a further means of differentiating *Escherichia* and *Aerobacter* strains.

A19. A Bacteriological Survey of a Swimming Pool Treated with Silver. W. L. MALLMANN, Department of Bacteriology, Michigan State College, East Lansing.

A bacteriological survey was made of a swimming pool treated with silver, introduced by the Katadyn process. All samples were collected in sodium-thiosulphate-treated sample bottles. Serial samples were collected during the actual period of bathing. Tests were made for total bacterial counts, streptococcus indices, and coli indices. The data show that silver is slower than chlorine in its bactericidal activity, hence bathing loads must be proportionately much lower. Three nonpathogenic bacteria that would grow in the presence of silver were found in the pool water.

A20. Optical Activity of Lactic Acid Produced by Lactobacillus acidophilus and Lactobacillus bulgaricus. LENORE M. KOPELOFF, NICHOLAS KOPELOFF, J. L. ETCHELLS AND E. POSSELT, Department of Bacteriology, New York State Psychiatric Institute and Hospital, New York.

The optical activity of the lactic acids produced by single strains of R and S forms of *Lactobacillus acidophilus* and *Lactobacillus bulgaricus* were studied according to the chemical methods of Pederson, Peterson, and Fred (1926). It was found that:

1. The R form of *L. acidophilus* produced inactive lactic acid. The R form of *L. bulgaricus* produced inactive lactic acid in the first six fractions, while the seventh yielded the dextrorotatory enantiomorph. The latter represented one-sixth of the total zinc salt.

2. The S forms of both *L. acidophilus* and *L. bulgaricus* produced dextrorotatory lactic acid.

3. It is suggested that some of the inconsistencies reported in the literature might be due to the use of cultures containing various combinations of R, S, and intermediate forms.

A21. The Gas-Producing Species of the Genus Lactobacillus. CARL S. PEDERSON, New York State Agricultural Experiment Station, Geneva.

The 19 gas-producing species of the genus *Lactobacillus* listed in the present (Fourth) edition of Bergey's Manual bear the same relationship to the non-gas-producing species of the genus *Lactobacillus* as does the genus *Leuconostoc* to the genus *Streptococcus*. Strains of only ten of these are available at present although isolations of strains similar to others have been made. Two additional species have been described recently. This group of 19 species is comparable in a general way to the two species included by Orla-Jensen in the genus *Betabacterium*. A study of the above mentioned cultures as well as several hundred additional cultures representing the various species have shown some differences between individual strains, but only a more or less natural variation when the entire group is considered. It is difficult to find any one character which can be used to divide the group into different species. There is little justification for the large number of species listed in the group at present. It is doubtful whether the entire group should be divided into more than four or five types which may be regarded as species.

A22. Surface Microflora of Limburger Cheese. C. D. KELLY, New York Agricultural Experiment Station, Geneva.

The typical limburger ripening is carried out largely by the enzymes of microörganisms growing on the surface of the cheese. In a study preliminary to an investigation of these organisms smears were made of the cheese in 14 New York State cheese factories by pressing microscopic slides on the outside of the cheese. These smears revealed the microbiological changes taking place from day to day.

For the first 24 hours the cheeses were found to have few organisms present and these were mostly budding yeasts, cocci and rods in about equal numbers. From the second or third day the budding yeasts were found to increase rapidly until they were present in large masses at four to five days. After the sixth day short slender rods appeared growing with the yeasts. These bacteria increased rapidly until at about eight days they were found in masses covering the cheese. From

ten to 18 days the yeasts decreased and on the older cheeses were seldom observed. A preliminary examination of isolated cultures of the slender rods indicated that this organism is *Bacterium linens* Weigmann.

Since these two types of microörganisms were found to the exclusion of all others on the better cheeses, it may be supposed that they are responsible for the surface ripening of this type of cheese.

Oospora lactis was found on some of the cheeses and where this organism was present in large numbers the surface of the cheese had a wrinkled appearance.

A23. Factors Affecting the Activity of Swiss Cheese Starter Cultures.
PAUL R. ELLIKER AND WILLIAM C. FRAZIER, Department of Agricultural Bacteriology, University of Wisconsin, Madison.

To determine the combined effect of the age of sterile reconstituted skim milk used and of incubation time and temperature of *Lactobacillus helveticus* (39aW), cultures were carried at both 37°C. and 40°C. in freshly prepared milk as well as in milk which had been allowed to stand at room temperature for seven days before use. The cultures were transferred daily and incubated for 12, 14 and 16 hours, respectively, after which they were kept in the icebox until the next transfer. The object of this procedure was to obtain cultures at 37°C. which were just as mature from the standpoint of acid produced as certain of the cultures grown at 40°C.

When the fresh medium was used, the activity of all of the cultures during a number of successive transfers steadily increased up to a certain point. Moreover their activity was decidedly greater than when they were grown in old milk. Steaming just before use apparently did not make old milk as favorable a medium as freshly autoclaved milk. To judge the activity of a culture, not only was rate of acid development determined, but the culture was also inoculated into a new lot of milk, heated in this milk at 60°C. for 30 minutes, then incubated at a temperature just below the maximum for the organism and tested for rate and amount of acid development and growth. When grown in fresh milk, cultures which had been carried at 37°C. were more active after heat-shocking than those which had been grown at 40°C. On the other hand when the older and apparently poorer medium was used, cultures which had been grown at 40°C. were more active following the heat treatment than those at 37°C.

These preliminary results indicate that a culture should reach a certain stage of maturity within a definite time if it is to be active

following the heat treatment. In a favorable medium this maturity may be obtained by carrying the culture at a temperature of 37°C., but in a poorer medium a slightly higher temperature may be preferable.

A24. A Bio-Physical Study of Oospora lactis. J. R. KURTZ, L. B. SCHWEIGER AND E. H. PARFITT, Purdue University, Lafayette, Indiana.

Morphological and physical characteristics of varieties of *Oospora lactis* isolated from butter have been studied from single cell isolations. Differences in the varieties isolated have been found in microscopic appearance. The hydrogen ion concentration of the substrate within the pH limits of 6 to 3 influences the rate of growth as measured by the dry weight of the mycelium and spore count. Distinct differences as to optimum growth temperature in the varieties isolated were found as measured by spore count. Several varieties were found to have higher thermal death points than the typical *Oospora lactis*.

A25. The Action of Air under Pressure in the Oxidation of Acetylmethylcarbinol to Diacetyl in Butter Cultures. C. R. BREWER, M. B. MICHAELIAN, C. H. WERKMAN, AND B. W. HAMMER, Sections of Bacteriology and Dairy Industry, Iowa Agricultural Experiment Station, Ames.

Previous work by Michaelian and Hammer (Ia. Ex. Sta. Bul. 205, 1936) has shown that the diacetyl content of skim milk cultures of the citric acid fermenting streptococci can be regularly increased by bubbling oxygen through the freshly acidified cultures.

In the present work, similar increases up to several hundred per cent in the diacetyl content of both pure cultures of citric acid fermenting streptococci and butter cultures were obtained by bubbling air through the cultures under pressure. Slight increases in the acetylmethylcarbinol content of the cultures were also found. Pressures up to 60 pounds per square inch were used.

It was found that the effect of pressure alone (without aeration) on the oxidation of acetylmethylcarbinol to diacetyl was negligible. Cultures saturated with oxygen failed to yield increases in diacetyl content over controls held at atmospheric pressure.

Experimental churnings of cream to which the cultures aerated under pressure were added, showed a consistent improvement in flavor and aroma over controls grown without aeration at atmospheric pressure.

A26. Reaction of Escherichia-Aerobacter from Milk on Eijkman Medium.
M. T. BARTRAM AND L. A. BLACK, Department of Bacteriology, University of Maryland, College Park.

Four hundred and fifty-four strains of coli-aerogenes organisms were inoculated from 24-hour agar slants into Perry and Hajna's modification of Eijkman medium and incubated at 46°C. (temperature of the media). The cultures had been isolated from milk three months to two years previous to the testing and included five species of *Aerobacter*, five species of *Citrobacter* and 14 species of *Escherichia*.

Of the 91 strains of *Aerobacter*, 87 or 95.5 per cent were negative (absence of gas within 48 hours), 127 or 98.5 per cent of the 129 intermediate strains were negative and 15 or 6.7 per cent of the 224 *Escherichia* strains were negative.

Three of the 45 strains of *Aerobacter hibernicum* and one of 24 strains of *Aerobacter cloacae* produced gas. One of 87 *Escherichia communior*, one of 23 *Escherichia paragruenthali*, one of 16 *Escherichia formica*, six of 15 *Escherichia enterica*, one of 13 *Escherichia gruenthali*, two of four *Escherichia pseudocoloides*, one of two *Escherichia neapolitana*, and the single strains of *Escherichia anaerogenes* and *Escherichia leporus* failed to produce gas in 48 hours.

None of the strains of *Aerobacter aerogenes*, *Aerobacter oxytocum* or *Aerobacter levans* were positive while gas was formed by all strains of *Escherichia coli*, *Escherichia vesculiformans*, *Escherichia anindolica* and *Escherichia pseudocoscorba*.

A27. Productivity of Media Used in the Isolation of Escherichia-Aerobacter from Milk. M. T. BARTRAM AND L. A. BLACK, Department of Bacteriology, University of Maryland, College Park.

The method described by Butterfield and Hoskins for determining the comparative productivity of media for coli-aerogenes was modified by using 10 instead of 15 tubes of each medium and by planting two dilutions instead of three. With solid media two dilutions, selected to yield 30 to 300 colonies per plate, were plated into each of the trial media in triplicate. In order to simulate conditions in previous experiments in which milk was used as the inoculum, one cubic centimeter of sterile whole milk was also added to each tube or plate.

The media employed were made so that the addition of the milk reduced the concentrations to the usual value. Two strains each of *Escherichia*, *Aerobacter* and intermediates recently isolated from milk were used. The dilutions were so made that one cubic centimeter

quantities were inoculated in each case and in all instances the media were inoculated alternately.

On the basis of the results obtained the liquid media were placed in the following order with the most productive first: (1) methylene-blue brom-cresol purple (2) fuchsin lactose (3) brilliant-green bile and (4) formate-ricinoleate. The Eijkman medium gave negative results in all tests. The solid media ranked as follows: (1) neutral-red bile (2) violet-red bile (3) Endo (4) brilliant-green lactose bile (5) lactose taurocholate (6) desoxycholate and (7) trypaflavine agar.

A28. Public Drinking Glass Sanitation in a Southern City. SETH T. WALTON, H. M. MORTON, AND MARY T. DAVIS, City Health Center, Charlotte, North Carolina.

In a bacteriological survey of public drinking glasses and rinse waters made in Charlotte, N. C., 252 glasses and 117 samples of rinse water were examined for the presence of total bacteria, hemolyzing bacteria, streptococci, organisms of the colon-aerogenes group and of Vincent's Angina. Soda fountains, cafes, and beer saloons were included in the survey.

Samples were collected at the establishment in sterile containers and transferred to the laboratory for examination. Counts in rinse waters ranged up to millions per cubic centimeter, including thousands of coli colonies. On glasses many thousands of bacteria per glass were recorded with positive tests for the colon-aerogenes group. Hemolytic streptococci and Vincent's organisms were frequently present.

As a result of the survey we were enabled to get a city ordinance passed recently requiring sterilization by chlorine, but to date no studies have been made to determine the efficacy of this method of sterilization.

A29. Reduction of Elemental Sulfur by Some Autotrophic and Heterotrophic Microöganisms. ROBERT L. STARKEY, Department of Soil Microbiology, Agricultural Experiment Station, New Brunswick, N. J.

The sulfur material precipitated by the bacterium *Thiobacillus thioparus* during its oxidation of thiosulfate, has been examined for the presence of sulfide, since it has been claimed that such material is a polysulfide. The results of gravimetric, volumetric, and colorimetric determinations were negative. Likewise no sulfide was found in cultures of another sulfur bacterium, *Thiobacillus thiooxidans*. During the growth of these two bacteria in inorganic media, sulfide was evolved

in small amounts as indicated by darkening of lead acetate test papers suspended over the culture solutions. In similar media containing elemental sulfur, sulfide was evolved by various heterotrophic organisms including bacteria, actinomycetes, and filamentous fungi. The results indicate that the sulfide formed by the autotrophic bacteria is a product of hydrogenation of elemental sulfur. This reaction points to the presence of active —SH groups in the sulfur bacteria and appears to have the same significance as hydrogenation of sulfur by heterotrophic microorganisms and other living cells. It is considered unlikely that elemental sulfur undergoes hydrogenation preceding its entrance into the cells of *Thiobacillus thiooxidans* which oxidize the sulfur to sulfate.

A30. Cellulose Decomposition by a Bacterial Culture from the Intestinal Tract of Termites. P. A. TETRAULT AND W. L. WEIS, Purdue University, Indiana.

Cellulose-decomposing bacteria were obtained from the gut of the common termite, *Riticulitermes flavipes*. The culture was enriched in Khouvine's nitrate cellulose medium to which 20 per cent yeast water was added. Isolations were made on this same medium without carbonate but with 0.8 per cent agar added and the solidified agar was covered with sterile vaseline. Colonies were picked into the enrichment medium and incubated at 37°C.

This purified culture contained two distinct morphological types: one, a long slender rod, occurring singly and in pairs measuring three-tenths microns by six to eight microns; the other was a large thick rod, in chains of three to six. It measured eight-tenths micron by two to four microns.

Two per cent cellulose was digested in from six to eight days. The products were acetic, lactic and butyric acids, in the order of greatest yield. Ethyl alcohol was produced in very minor quantities. A trace of some reducing substance was always present. The gas was mostly carbon dioxide.

A31. Bacteria and the Nitrogen Metabolism of Termites. ROBERT A. GREENE AND EDWARD L. BREAZEALE, Arizona State Laboratory, Tucson.

Bacteria were isolated from an unidentified species of *Kalotermes*, which, when inoculated into a nitrogen-free mannitol solution, fixed quantities of nitrogen varying from 0.4 to 1.3 mgm. The conditions in the intestinal tract of termites, because of the wide C:N ratio should

favor the fixation of gaseous nitrogen. Since the nitrogen requirements of termites are low and the average nitrogen content of the termites studied was 0.862 mgm., it seems probable that microörganisms may play an important rôle in the nitrogen metabolism of termites.

A32. Digestion of Corn-Cobs by Bacteria. P. A. TETRAULT AND J. HURWITZ, Purdue University, Indiana.

The bacterial culture used in this work was obtained originally from the gut of a termite. It was enriched in Khouvine's nitrate cellulose media to which 20 per cent yeast water was added. Purification was accomplished by (1) the dilution method, (2) pasteurization and (3) growing at 45°C. The resulting culture was very active and could digest corn-cobs.

Best results were obtained when the cobs were immersed in water and autoclaved at 15 pounds for 45 minutes. The water was filtered off and the cobs were dried at 105° for 48 hours. These treated cobs were incorporated into the medium and inoculated with an active culture. The fermentation at 37° was complete in six to eight days. The products acetic, butyric and lactic acids were recovered. Ethyl alcohol was not found.

A33. Effect and Efficiency of Germicides and Fumigants on Microörganisms Associated with the Baking Industry. GERALD K. ASHBY, C. C. HEDGES AND E. H. GIBBONS, Departments of Chemistry and Biology, Agricultural and Mechanical College of Texas, College Station.

In a study to determine the effect of germicides and fumigants on bacteria that produce "rope" in bread, five species of the genus *Bacillus*, proven to produce "rope" in bread, were used. Spores of these organisms, suspended in veal infusion, wheat flour suspension, and in saline, were exposed to: hypochlorites; formaldehyde; acetic acid; and phenol. Spores of three of these species were also exposed to hypochlorite spray and to formaldehyde gas.

Five per cent phenol was found to be ineffective. In disinfectant solutions containing wheat flour or saline, hypochlorites were very effective while formaldehyde and acetic acid were not. Not only hypochlorites, but also the other disinfectants were ineffective in solutions containing veal infusion.

As fumigants, the hypochlorites seemed to be ineffective while formaldehyde, if used in strong enough concentrations, was very effective.

For a study of the protective action of wheat flour and of fat in fumigation, contaminated specimens of wood, web belting, and of metal were heavily coated with wheat flour and with melted shortening. No protective action of the wheat flour could be demonstrated, but shortening afforded the test organisms considerable protection against the fumigants.

From a comparison of the relative strengths of the germicides as used in solution and as fumigants, it appears that hypochlorites may be effective in spray form if strong enough solutions can be used without causing corrosion of the materials sprayed.

A34. The Saprophytic Digestion of Heat-Sterilized Animal Hair and Keratose. L. S. STUART, Bureau of Chemistry and Soils, U. S. Department of Agriculture.

The most common fungi that developed on the hair of salted heavy hides were *Aspergillus glaucus* and *Chaetomium globosum.* Studies were made to determine the ability of these organisms to digest heat-sterilized animal hair and keratose.

Chaetomium globosum was found to utilize heat-sterilized animal hair as a sole source of carbon and nitrogen, and to bring about a reduction of the sulphur groups of keratose simultaneously with an increase in free COOH groups.

Aspergillus glaucus did not utilize heat-sterilized animal hair as a sole source of carbon and nitrogen, but did attack hair after it had been acted upon by bacteria or in the presence of glucose.

Results suggest that the digestion of animal hair keratin by microorganisms depends upon their ability to reduce the disulfide linkage.

A35. Materials Manufactured by Microörganisms. J. R. SANBORN, Research Division, International Paper Company, Glens Falls, New York.

Synthesis by microörganisms of industrially useful gummy materials offers potential commercial possibilities not yet evaluated. Various types of manufactured products such as semi-transparent sheets, adhesives, plastics, possible food ingredients and feedstuffs, add to the industrial significance of these processes.

Two groups of economically useful gum-formers are under investigation. The first, represented by *Oidium* and *Mucor*, grows abundantly in potato or cereal mashes containing glucose; other types, such as *Trichoderma lignorum*, appear to grow best in mineral solutions containing sucrose.

A preferred species of *Oidium* grows in rank profusion in starchy media containing glucose, mannose, glycerol, or ethyl alcohol. Additional sources of nitrogen such as ammonium nitrate, peptone, or gelatin, do not cause appreciable increases in yields of *Oidium* gum. While mineral solutions containing utilizable carbohydrates are generally unsatisfactory for commercial cultivation of *Oidium*, potato decoction (Fritz) provides an excellent basis for large scale production. Slops from starch manufacture present significant though somewhat less favorable possibilities.

Growths allowed to develop undisturbed produce larger total yields of gum than those obtained by removal of successive crops.

A36. The Action of Microörganisms on Fats. L. B. JENSEN, Swift and Company, Chicago, Ill.

A study is being made of: (*a*) Culture methods to demonstrate lipoclastic actions of microörganisms, (*b*) kinds of microbial actions on fats, (*c*) diffusion of chromogenic substances of microörganisms into fats, (*d*) action of bacterial lipases and oxidases on fats stored at 32°F. for one year, and (*e*) action of microörganisms on pure fats.

Observations of growth on Eijkman's medium, Lieske's emulsion agar, Turner's cottonseed oil-Nile blue agar, cocoanut oil agar, palm oil emulsion agar, and other media of this type point out lipoclastic actions in some instances. If mineral oil is substituted for digestible oils in these emulsion agars to which fatty acid staining dyes are added, colonies are often observed similar to those considered lipoclastic on vegetable or animal fat emulsion agars. This phenomenon appears to be due to bacteria concentrated in the interfacial trap in the oil-water emulsion agar and affecting dye.

The data, then, cannot be obtained from emulsion agar media, but must be obtained by inoculating washed microbes into commercial fat. After incubation, inoculated fats are tested: (1) By an accelerated-rancidity test for critical peroxide value, (2) for free fatty acid, (3) by aldehyde and ketone tests, (4) by the Kreis test, and (5) by organoleptic test. These tests show that bacteria may induce: (*a*) Oxidative rancidity (lipase-peroxidase formers), (*b*) hydrolysis with high free fatty acid (lipase formers), (*c*) tallowiness (oxidizers) in beef and mutton fats, and (*d*) flavor reversion and production of flavor adjuvants (organoleptic).

Fat-soluble pigments of various microörganisms cause "pink" fats and purple "stamping ink" discolorations by oxidation-reduction mechanisms.

Killing fats, cutting fats, sweet pickle fats, and mixed killing and cutting fats, inoculated with oxidase-lipase bacteria, after storage for one year at 32°F., show peroxide values in milliequivalents per kilogram of 1.4 to 2.4 (controls) to 12 to 50 (rancid, inoculated samples). Pure fats, as free as possible from moisture, apparently do not support any type of bacterial, mold, or yeast growth.

A37. Observations on Methods for the Study of Lipolysis by Microörganisms. H. F. LONG AND B. W. HAMMER, Iowa State College, Ames.

The ability of a microörganism to hydrolyze fat is an important biochemical character and one that is of value in the identification of species.

Various investigators have suggested procedures which differ in their usefulness for detecting lipolysis by organisms. The addition of Nile blue sulfate and dispersed fat to a medium is a simple procedure and gives a clear cut reaction, but the inhibitory effect of the dye is a disadvantage in some instances. However, because of the inhibition of many non-lipolytic organisms, especially cocci, the Nile blue sulfate technic facilitates the enumeration and isolation of lipolytic species when they are present in small numbers as compared to the total numbers. When a medium containing dispersed fat is flooded with Nile blue sulfate after incubation, the results are easily read and total as well as lipolytic counts can be obtained; but the picking of lipolytic colonies is complicated by the washing of organisms over the surface of the medium. The lower simple triglycerides (e.g., tripropionin or tributyrin) are more easily hydrolyzed than natural fats; therefore the results obtained with them may be misleading. The natural-fat technic, in which lipolysis is detected by a change in the opacity of the fat, is valuable, because in addition to permitting total and lipolytic counts on the plates the lipolytic organisms are easily isolated.

A38. Reproduction of Yeast Cells in Various Nutrient Solutions. (*With motion pictures.*) CHARLES N. FREY, The Fleischmann Laboratories, New York City.

This film shows the reproduction of yeast under normal conditions. Growth and general characteristics under certain abnormal conditions are also shown. The lack of proper nitrogenous material, deficiencies in phosphorus, magnesium, and potassium were investigated. The effect of a number of factors influencing growth and reproduction of yeast has been studied.

A39. Effect of Various Factors on the Vitamin B_1 Content of Yeast.
P. L. PAVCEK, W. H. PETERSON, AND C. A. ELVEHJEM, Departments of Agricultural Chemistry and Bacteriology, University of Wisconsin, Madison.

An apparatus in which kilogram batches of yeast can be grown aseptically has been devised. About 60 such batches have been grown, with variations in the type of medium, strain of yeast, and certain conditions of growth (pH, temperature, aeration, etc.). Three types of media (grain wort, molasses-salts, glucose-salts) and six types of yeast (two bakers' yeasts (*Saccharomyces cerevisiae*), one brewers' yeast, *Saccharomyces logos, Willia anomala,* and *Torula galactosa*) were used. The yields of dry yeast, based on sugar fermented, in the wort medium ranged from 24 per cent (bakers' yeast B) to 40 per cent (bakers' yeast A); in the molasses medium from 28 per cent (*Saccharomyces logos*) to 43 per cent (brewers' yeast); and in the glucose-salts medium from 12 per cent (*Willia anomala*) to 29 per cent (brewers' yeast). The effect of omitting aeration, raising the pH (6.0 instead of 4.3) and of lowering the temperature (20° instead of 30°) was studied for one of the bakers' yeasts. All of these changes decreased the yield, but lack of aeration produced the most marked effect.

The vitamin B_1 content of the various batches of yeast was determined by biological assay with one-day-old chicks as test animals. Bakers' yeast contained the largest amount of vitamin when grown on wort medium, approximately 10 I. U. (International Units) per gram of dry yeast; on molasses-salts the figure was 5 I. U., and on synthetic medium the vitamin content decreased to 3 I. U. (Commercial bakers' yeast assayed 5 to 13 I. U.). When brewers' yeast, which ordinarily contains about 50 I. U., was propagated on the above three media, the vitamin content became about the same as that for bakers' yeast on the corresponding media. Other strains of yeast also assayed about the same as bakers' yeast.

From these data it appears that composition of the medium is the most important factor in determining the vitamin B_1 content of yeast.

A40. Bacterial Dissimilation.
C. H. WERKMAN, R. W. STONE AND H. G. WOOD, Department of Bacteriology, Iowa State College, Ames.

Bacterial dissimilation of glucose is characterized by phosphorylation in the initial phase. The evidence supports phosphorylation by the living cell. This assimilatory stage in the initial phase of the general

phenomenon of dissimilation has been shown with: *Clostridium*, *Aerobacter*, *Citrobacter*, *Escherichia*, *Lactobacillus*, *Propionibacterium*, *Bacillus* and *Serratia*, among others. The hexose phosphate is probably an equilibrium mixture of the mono- and di-esters. Additional evidence is needed.

Re-evaluation of the rôle of methylglyoxal, generally accepted as the key intermediary in bacterial metabolism is now necessitated by our isolation of phosphoglyceric acid from the dissimilation of glucose and hexose diphosphate by a wide variety of microörganisms: (from hexose diphosphate + glucose) *Propionibacterium shermanii*, *Propionibacterium arabinosum*, *Propionibacterium pentosaceum*, *Lactobacillus pentoaceticus* (heterofermentative), *Lactobacillus plantarum* (homofermentative), *Escherichia coli*, *Citrobacter freundii*, *Aerobacter aerogenes*, *Bacillus subtilis*, and *Serratia marcescens*.

Phosphoglyceric acid was isolated in the presence or absence of added acetaldehyde, when pyruvic acid replaced acetaldehyde as a hydrogen acceptor, or small yields occurred in the absence of toluene.

Phosphoglyceric is transformed into pyruvic + phosphoric acid. Conversion is slow and details of the intricate mechanism await explanation; the behavior of adenylic acid appears to be important. Pyruvic acid has been isolated as an intermediary with *Propionibacterium*, *Escherichia*, *Aerobacter*, *Lactobacillus*, *Clostridium* and *Citrobacter*.

The final phases of dissimilation involve delicately balanced oxidation-reduction equilibria or easily diverted hydrogen transferences.

A41. Phosphorylation and First Stages in Glucose Breakdown by Propionic Acid Bacteria. R. W. STONE, H. G. WOOD AND C. H. WERKMAN. Department of Bacteriology, Iowa State College, Ames.

Suspensions of propionic acid bacteria cause phosphorylation of glucose in the presence of the dephosphorylation-inhibiting substance NaF. This takes place either with or without the presence of toluene. The phosphate ester, phosphoglyceric acid, has been isolated from such fermentation mixtures. A small amount of this ester can be isolated without any NaF present, but toluene or some other poison is necessary. The amount of phosphate uptake in the presence of such poisons as NaF is many times greater than the equivalent of phosphoglyceric acid obtained. This suggests that most of the phosphate is retained in one or more hexose or triose esters, presumably precursors of phosphoglyceric acid. The rôles of phosphorylation, methylglyoxal, and phosphoglyceric acid in the propionic fermentation are discussed.

A42. The Final Oxidation-Reduction Phases of the Propionic Dissimilation. H. G. WOOD, R. W. STONE AND C. H. WERKMAN, Department of Bacteriology, Iowa State College, Ames.

Pyruvic, lactic, acetic and succinic acids are proposed as intermediates in the dissimilation of glucose by propionic acid bacteria. All have been isolated by special fixation methods and are utilized by the organisms. A scheme of dissimilation is presented involving a conversion of the hexosephosphate into phosphoglyceric acid which in turn yields pyruvic acid. Pyruvic acid is oxidized to acetic acid and CO_2 and reduced to propionic acid through lactic. Succinic acid is probably formed from acetic acid and in turn is dissimilated to propionic acid and CO_2. The assimilation of CO_2 to a fermentable unknown compound has been established in glycerol dissimilation. The rôle of methylglyoxal is discussed.

A43. Mechanism of the Formation of Isopropyl Alcohol by Clostridium butylicum. A. F. LANGLYKKE AND E. B. FRED, Departments of Agricultural Bacteriology and Chemistry, University of Wisconsin.

In the fermentation of glucose by a strain of *Clostridium butylicum* acetone accumulated before the production of isopropyl alcohol was noted. This suggests that isopropyl alcohol is formed through the reduction of acetone. When added to the fermentation, acetone was reduced to isopropyl alcohol.

The addition of pyruvic acid to the fermentation did not result in an accumulation of the corresponding reduction product, lactic acid, but gave products normal to the fermentation. Acetylmethylcarbinol was quantitatively reduced to 2,3-butylene glycol, and acetaldehyde was reduced to ethyl alcohol.

The addition of compounds which serve as hydrogen acceptors always caused increased synthesis of isopropyl alcohol and acetone from the carbohydrate and decreased formation of butyl alcohol and butyric acid. The explanation proposed is that the utilization of hydrogen by the foreign compounds causes a decrease in the hydrogen available for the usual reductive processes. Consequently, those compounds, isopropyl alcohol and acetone, which arise through decarboxylation reactions are produced in greater proportion than those, butyl alcohol and butyric acid, which require hydrogenation reactions.

A44. The Fermentation Products of Clostridium thermosaccharolyticum. N. O. SJOLANDER, ELIZABETH MCCOY AND L. S. MCCLUNG, University of Wisconsin and University of California.

Clostridium thermosaccharolyticum has been described by McClung as an anaerobic thermophile, which produces acid and gas from a large number of carbohydrates. More definite information on the products of fermentation is desirable, because of the importance of this organism in spoilage of canned food.

Various basal media were tried and maximum utilization of glucose was obtained in 2 per cent malt sprouts and in 1 per cent tryptone with asbestos, both containing an excess of calcium carbonate. In these media, glucose in concentration of 2 per cent could be completely utilized.

The products of the dissimilation of glucose were carbon dioxide, hydrogen, acetic acid, butyric acid, and lactic acid. The lactic acid was identified by preparation of the para-phenyl-phenacyl ester and the zinc salt, which had three molecules of water of crystallization corresponding to that of inactive zinc lactate. In a typical experiment in which all products were determined, 100 millimols of glucose yielded 238 millimols of hydrogen, 175 millimols of carbon dioxide, 60 millimols of butyric acid, 48 millimols of acetic acid, and 26 millimols of lactic acid. In this fermentation the weight balance was 103.4 per cent; the carbon balance was 98.0 per cent; and the oxidation-reduction balance was 1.008. This ratio of products is apparently influenced by conditions of temperature and oxygen tension. On xylose this organism formed the same products in somewhat different proportions.

A45. Some Characteristics of an Organism Causing Spoilage in Fortified Sweet Wines. H. C. DOUGLAS AND L. S. MCCLUNG, University of California, Berkeley.

In 1933 a peculiar type of spoilage of sweet wines, not previously seen in California wines, was observed by W. V. Cruess. The spoilage, which has been serious, has been found most frequently in muscatel, sherry, and angelica wines, and occasionally in port, Tokay and Malaga. It is characterized by an extensive flocculent albumin-like sediment. Microscopic examination of such material has revealed long intertwined filaments of the uniform width of about one micron. These filaments appeared segmented but no branching has been observed. The majority of the filaments are Gram-negative, although Gram-positive segments have been observed. Spores have not been demonstrated. Attempts to cultivate the organism in all of the usual laboratory media with various conditions of incubation have met with complete failure. Growth has resulted only with the use of sweet wine diluted with an

equal volume of water and sterilized by steaming. Dilution tubes of agar of this medium have revealed that anaerobic conditions are essential for growth. The optimum pH range in the liquid medium has been found to be 4.1 to 4.3 and the optimum temperature 20 to 25°C. Chemical analyses have shown that on prolonged incubation the volatile and fixed acids of the wine are increased and the reducing sugars are destroyed. Slight gas formation has been noted in some instances and the pH of the wine has decreased. Although the majority of the reports of this spoilage has been in bottled wine positive cultures have been obtained from storage vats indicating that in some instances the wine is contaminated in the winery before bottling. Investigation of control measures has shown that either pasteurization or sulfur dioxide may be successfully applied.

Additional physiological characteristics of this organism are being studied in an effort to determine the systematic position and the relationship to previous types responsible for spoilage of wine.

A46. Proteolysis by Mold Enzymes. JULIUS BERGER, M. J. JOHNSON AND W. H. PETERSON, Departments of Agricultural Chemistry and Agricultural Bacteriology, University of Wisconsin, Madison.

The extent of proteolysis attained by enzyme mixtures from animal sources has been shown by other workers to be about 85 to 90 per cent of the amount possible by acid hydrolysis. A preliminary experiment had shown that gelatin was hydrolyzed by mold enzymes only to the extent of 60 to 70 per cent. In order to determine whether this was due to the nature of the protein alone or to the absence of certain enzymes in the proteolytic enzyme complex of molds, other proteins were hydrolyzed by mold enzyme preparations. Lactalbumin, egg albumin, edestin, casein, gliadin and zein were hydrolyzed by a dialyzed enzyme preparation from *Aspergillus parasiticus* to the extent of approximately 85 per cent after 20 to 30 days' incubation.

Gelatin solutions ranging in concentration from 0.5 to 16 per cent were subjected to the action of *Aspergillus alliaceus* enzyme. It was found that the lower concentrations of protein were hydrolyzed more rapidly and to a greater extent than the higher concentrations.

It was also found that when *Aspergillus alliaceus* gelatin hydrolysates were further incubated after addition of hog erepsin or *Aspergillus parasiticus* enzyme, only very slight further hydrolysis took place.

A47. The Variability of Crop Weight Determinations in Liquid Mold Cultures. KARL J. DEMETER AND MAX LOEWENECK, Bacteriology Department, South German Research Institute for Dairying, Weihenstephan-Munich. (Read by title.)

It has been shown by us and by others that the growth of parallel cultures of molds (penicillia) in liquid media is very unequal. This is true, if in all culture flasks the conditions for growth are strictly the same, including the amount of spores used for inoculation.

Experiments were conducted by culturing penicillia in 16–17 parallel culture flasks simultaneously, under the same conditions, and analyzing the crop weight of one set after a certain period of days and of the second set one day later.

The differences found in the crop weight of the parallel cultures were considerable, e.g., (values in milligrams): Crop weights of *Penicillium candidum* from seven day and eight day cultures showed mean deviations of ±86 and ±56, respectively, and coefficients of variation of 79 and 25, respectively. Crop weights of *Penicillium camemberti* from six day and seven day cultures showed mean deviations of ±54 and ±20, respectively, and coefficients of variation of 38 and 7, respectively.

Since many crop weight determinations by other workers with liquid media have been based usually on not more than one or two parallel cultures, it is clear that such values are accidental and not fit for comparative investigations on the physiology of the nutrition of fungi. It is interesting to note how decidedly the results improve, if the cultures are only one day older.

That the results could be influenced readily was shown by other experiments in which the cultures had been lightly shaken once on the first and once on the fourth day after inoculation. The crop weights were lowered to one-third to two-thirds of the values for the unshaken cultures.

To surmount these difficulties experiments were started with solid media, which were, however, not so solid as to check loosening of the mycelia. A synthetic one per cent agar medium proved satisfactory. Using a certain technique it was easy to get off the mycelium quantitatively and free it from the adhering agar particles. The first set of experiments with a mean crop weight of 256 mgm. resulted in a mean deviation of only 10 mgm. with a coefficient of variation of only 4.

INDEX OF AUTHORS

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

EASTERN NEW YORK BRANCH

State Department of Health Laboratories, Albany, October 30, 1936

The Anticoagulative Action of Organic Acids and of Heparin and the Organic Base Diethylamine. *Augustus Wadsworth and Frank Maltaner* (by invitation), Division of Laboratories and Research, New York State Department of Health, Albany.

Organic acids inhibited the coagulation of plasma to a degree directly proportional to their titratable acidity. Molecular solutions of citric acid were three times, and those of oxalic or succinic acid twice, as active as lactic, pyruvic or galacturonic acids. Carboxy or sulphonic acids were equally effective. Monocarboxy amino acids were inactive, whereas dicarboxy amino acids inhibited coagulation to the same degree as monobasic acids. The ureides, alloxan and parabanic acid, titrated as monobasic acids; they acted like the monocarboxy acids, as did cysteine hydrochloride. The inhibiting doses of these organic acids and dium oxalate were chemically equivalent. An equivalent amount of sodium hydroxide neutralized the anticoagulative action of all but oxalic acid, including cysteine hydrochloride.

Heparin, ten to twenty times more active than the organic acids, was not neutralized by sodium hydroxide but was neutralized by hydrochloric acid or, in the presence of calcium chloride, by cephalin in directly proportional amounts. Heparin prevented the reaction between cephalin, protein, and calcium chloride.

The organic base diethylamine was twice as effective in inhibiting clotting as the organic acids and its effect was neutralized by hydrochloric acid and by cephalin and calcium chloride in the same manner and degree as was heparin.

Horse and Rabbit Serum in the Complement-fixation Reaction. *Christine E. Rice*, Division of Laboratories and Research, New York State Department of Health, Albany.

At no stage during immunization do antipneumococcus or antimeningococcus horse sera fix complement with the respective homologous specific polysaccharides. Early in the process of immunization antipneumococcus horse serum, still of low precipitative titer, fixes complement with extracts of acetone-treated whole cells, nucleoprotein and lipid fractions of pneumococci. Later in the course of immunization, horse serum of high precipitative titer usually fixes complement with the lipid substances only. Immune horse serum, or normal horse serum in larger amounts, when added to systems containing immune rabbit serum and specific carbohydrate increases the degree of hemolysis.

Similarly, if normal horse serum, or more particularly the euglobulin fraction prepared from it, is added before

or immediately after the addition of complement to such particulate, anticomplementary, carbohydrate substances as agar or pectin, the hemolysis of red cells proceeds. Normal rabbit serum does not have this effect. Agar-treated complement is slowly reactivated by heated or unheated horse serum; the hemolytic activity of complement lost through treatment with ammonia is restored by the addition of small amounts of unheated rabbit serum or agar-inactivated guinea pig serum, but not by horse serum.

A COMPARATIVE STUDY OF THE ALCOHOL-ETHER AND THE ETHER-EXTRACTION METHODS FOR THE DETERMINATION OF LIPIDS IN SERUM. *L. W. Hyman and R. R. Nichols*, Division of Laboratories and Research, New York State Department of Health, Albany.

Analyses were made of the solids extractable from 4 cc. of horse serum by 100 cc. of alcohol-ether mixture. The total solids were determined by evaporation of 100 cc. and the residue ignited to give the organic solids. The inorganic salts, chiefly sodium chloride, were corrected for phosphorous. The dry residue from 50 cc. of the serum extract was treated with distilled water and hydrochloric acid and extracted with five successive 10-cc.-portions of ether. The total ether-extractable lipids, determined after evaporation and drying, were about one-fourth the amount of the solids in the serum extract. The difference consists of inorganic salts and nonlipid organic compounds soluble in alcohol-ether. The ether-extractable lipids approximated closely the sum of the cholesterol esters, free cholesterol and phospholipids determined in the serum extract.

In nonimmunized horses and in those under immunization for a long time, the nonlipid organic material was quite constant; however, it was considerably lower with streptococcus and pneumococcus horses under immunization for less than a year.

Since residues from the serum extract contain inorganic and organic nonlipid substances the direct method is incorrect. The ether extraction procedure proved a satisfactory gravimetric method for the determination of lipids in serum.

PROBLEMS IN THE FIXATION OF ATMOSPHERIC NITROGEN BY AZOTOBACTER AND ALLIED FORMS. *Carye P. Haskins.*

A SIMPLE TECHNIC FOR THE ULTRAFILTRATION OF BIOLOGICAL MATERIALS. *James J. Quigley and Gretchen R. Sickles*, Division of Laboratories and Research, New York State Department of Health, Albany.

Bacterial-specific carbohydrate and toxins have been purified by a method of ultrafiltration devised to filter large volumes at room temperature without contamination.

An alumina candle is coated with nitrocellulose following a method previously described. The candle is mounted and sealed in a metal holder with beeswax-paraffin mixture and locked with Mandler screws in an 11-by-2¾ inch pyrex glass mantle. A siphon from a 4-liter bottle is connected by rubber tubing to 5-mm. glass tubing, of sufficient length to reach the bottom of the glass mantle, inserted through a No. 14 rubber stopper fitted with an air-guard. The siphon, previously sterilized, is placed in a bottle of distilled-water sodium-hypochlorite solution containing 25 parts per million of available (free) chlorine; the bottle is elevated, the rubber stopper inserted

into the glass mantle and a bottle to catch the material from the filter is attached by pressure tubing to the end of the mounted nitrocellulose candle. The coated candle is sterilized by drawing the chlorine solution, followed by distilled water, through the filter. The siphon is then transferred to a bottle of the product to be filtered; the material is siphoned into the mantle and the lower bottle evacuated.

THE PHOSPHATASE TEST AS AN INDICATION OF PASTEURIZATION. *F. W. Gilcreas and W. S. Davis*, Division of Laboratories and Research New York State Department of Health, Albany.

The progressive inactivation of the enzyme phosphatase present in raw milk by heating to the time and temperature of pasteurization has been used by Kay and Graham (Journ. Dairy Research, 1935, **6**, 191–203) as the basis of a test for adequate pasteurization. If the sample is adequately buffered and incubated overnight at 37°C., the enzyme will hydrolyze an added phenyl - phosphoric - ester - liberating phenol, which is readily detected quantitatively by the use of Folin's reagent. For the tintometer used by the authors, however, a method of reading the characteristic color reaction of phenol in comparison with permanent standards prepared from inorganic salts has been developed. Variations of five minutes or greater in the heating time were readily distinguished and the addition of as small a quantity as 0.1 per cent of raw milk gave a result indicative of incomplete pasteurization. Variations in temperature were also easily detected. The technic proved equally satisfactory in determining pasteurization by the ordinary procedure and by the high temperature process.

The test has been applied to the examination of approximately 100 samples of milk representing varying conditions of temperature and holding time in commercial pasteurizing plants. A correct evaluation of the character of the treatment to which the milk had been subjected was made in 97 per cent of the specimens. If this precision can be maintained in extended routine practice, the test should prove an invaluable aid in the control of pasteurization.

AN EXTENSIVE OUTBREAK OF ENTERIC DISEASE INCITED BY B. DYSENTERIAE, SCHMITZ. *J. Schleifstein and M. B. Coleman*, Division of Laboratories and Research, New York State Department of Health, Albany.

Bacillus dysenteriae Schmitz was found to be the incitant of an extensive epidemic of enteric disease in a New York state institution in August and September, 1936. More than 200 cases were reported among over 5,000 inmates and employees; this species was isolated from the feces of twenty-nine individuals. All of the cases were relatively mild. In two instances, a temperature of 104°F. was recorded, but the reports indicated that in most of the cases evidence of little if any elevation of temperature was found. The onset was sudden, with abdominal pain followed by diarrhea with blood and mucus which persisted for two or three days.

This type of dysentery bacillus has apparently been recognized very rarely as an etiological agent of enteric disease in the United States. It was the incitant of a small outbreak which occurred in 1934 in another institution in New York State and has also been found in fecal specimens examined in the Division of Laboratories and Re-

search from a few isolated cases during the past three years.

PRESERVATION OF SPUTUM SPECIMENS FOR PNEUMOCOCCUS-TYPE DIFFERENTIATION. *A. H. Harris and F. M. Varley*, Division of Laboratories and Research, New York State Department of Health, Albany.

The preservation of sputum from pneumonia patients by the addition of 1-per-cent formalin has been found advantageous in preparing specimens for prolonged use in teaching and for exhibition purposes.

A series of nineteen specimens was studied; ten contained type-I pneumococci, two type-II, three type-III, three type-VIII, and one type-XIV. The formalin solution was found to be very effective in preserving both the cellular structure and the "*Quellung*" capacity of the pneumococci except in the case of type III.

The method used consisted of adding to a portion of sputum a 1-per-cent solution of formalin in an amount equal to at least twice that of the specimen and storing the mixture at room temperature.

CONNECTICUT VALLEY BRANCH

YALE MEDICAL SCHOOL, NEW HAVEN, CONNECTICUT, NOVEMBER 14, 1936

OXIDATION-REDUCTION POTENTIAL STUDY WITH THE AEROBACILLUS GENUS. *J. Roger Porter and Russell W. H. Gillespie*, Department of Bacteriology, Yale University, New Haven, Connecticut.

The genus *Aerobacillus* was created in 1926 by Donker, to include all sporulating, facultative anaerobic bacteria which produce catalase and are able to produce acid and gas from various carbohydrates. Five species were recognized by Donker.

The genus has been reported (Porter, McCleskey and Levine) to be rather sharply divided into two definite species, *Aerobacillus polymyxa* and *Aerobacillus macerans*, on the basis of certain cultural, physiological and serological characteristics. These results suggested a study of oxidation-reduction potentials of strains representative of the two species. According to our observations the two groups or species exhibit stable potentials at two widely different levels of reducing intensity. The grouping established potentiometrically coincides exactly with that previously established on cultural, fermentative and serological grounds. Consideration of the experimental evidence suggests the possibility that differences in carbohydrate metabolism may be responsible for the difference in reduction potentials. This is the fourth bacterial genus with which similar correlations have been established in our laboratory. The results of these experiments are such as to direct the course of further investigation into the significance of bacterial oxidation-reduction potentials.

LISTERELLA INFECTION ASSOCIATED WITH OVINE ENCEPHALITIS. *Erwin Jungherr*, Department of Animal Diseases, Storrs Agricultural Experiment Station, Storrs, Connecticut.

Five cases of meningo-encephalitis associated with *Listerella* infection were observed in a flock of 200 purebred sheep. Diagnosis was based in 1 instance on clinical examination alone, in 2 on histologic and bacterioscopic evidence, and in 2 entirely on cultural isolation of a *Listerella* organism from

the affected areas. Pathologically the disease was characterized by mononuclear infiltrations in the regional meningi and by perivascular areas and central polynuclear foci in the medulla oblongata. Similar disturbances were produced in mice by intranasal injection of brain suspension and small doses of *Listerella* culture, while filtered brain material was innocuous. Comparatively large culture doses injected intranasally produced in mice a rapidly fatal septicemia, and in sheep a marked thermic and agglutinative response followed by recovery Sheep receiving intracarotic injections of *Listerella* culture succumbed to hemorrhagic meningitis Primary isolation of the *Listerella* organism from field cases was effected on blood sugar agar slants and egg meat medium; subcultures on liver agar plates produced a characteristic milky, slightly opalescent, round, entire colony. Except for minor differences in delayed biochemical reactions, the ovine strains could not be distinguished from 2 human and 1 ovine reference strains by morphologic, cultural, agglutination and agglutinin absorption tests.

FILTRATION EXPERIMENTS WITH MYCOBACTERIA AND WITH A RECENTLY DESCRIBED ACTINOMYCES-LIKE ORGANISM. *Harriette D. Vera*, Department of Bacteriology, Yale University, New Haven, Connecticut.

Three strains of the human tubercle bacillus, 2 of the bovine tubercle bacillus and 9 soil strains of acid-fast bacteria were grown in Long's medium at room temperature and at 37.5°C. and filtered through Chamberland L3 candles. The cultures were filtered at ages varying from 1 day to 3 months. Control filtrations were made with sterile medium. All filtrates were closely observed over long periods of time. About 1100 transfers were made from filtrates to suitable media. Twenty filtrates (*Mycobacterium tuberculosis-hominis*) were washed forward 586 times in Blake bottles, according to the Hauduroy technique. 134 filtrations of cultures and 88 control filtrations were performed. None of the filtrates or transfers showed any evidence of growth of mycobacteria.

Similar experiments were carried out with the actinomyces-like organism described by Colien in 1935. This organism was grown in nutrient broth and in antiserum broth at room temperature and at 37.5°C. The ages of the filtered cultures were from 10 days to 6 months. Altogether, 50 cultures were filtered 112 times, with 96 control filtrations, and about 400 transfers were made. The organism was recovered from one filtrate. This single positive result was not considered significant as possible evidence of filterability, because it lay so definitely within the limits of experimental error. Moreover, neither refiltration of this nor of the original culture gave positive results.

IMMUNIZATION AGAINST TETANUS. *Philip B. Cowles*, Department of Immunology, Yale University, New Haven, Connecticut.

Questions pertinent to the active immunity of man to tetanus are: (1) How rapid is the response of an immune person to a subsequent injection of toxoid? and (2) What antitoxin titer in the serum can be considered adequate for protection? For the second of these, animal experiments must of course be used to suggest an answer.

Twelve men immunized a year previously and having titers ranging from $< \frac{1}{50}$ to $\frac{1}{5}$ unit of antitoxin per cubic centimeter of serum received 0.5 cc. of alum-precipitated toxoid subcu-

taneously. Six of these, when tested on the third day after toxoid administration, failed to show any significant increase in titer. Of eight tested on the fourth day, four responded. Of eight tested on the fifth day, seven had $\frac{1}{20}$ unit of antitoxin or more. By the seventh day all had reached $\frac{1}{5}$ unit or more, with the exception of one man who had only $\frac{1}{20}$ unit. The speed of response was apparently rather slow.

In actively immune mice titers of $\frac{1}{10}$ unit were the smallest to protect against spore-$CaCl_2$ injections which were fatal to controls in 2-4 days. All of 26 actively immune guinea pigs, none of which had titers of less than $\frac{1}{5}$ unit, were protected against an infection which was fatal in 2 days to all but 2 of an equal number of controls.

COMPARATIVE VALUES OF CLINICAL AND POSTMORTEM BLOOD CULTURE STUDIES. *Caspar G. Burn and Daniel F. Harvey*, Department of Pathology, Yale University, New Haven, Connecticut.

Complete agreement between clinical and postmortem blood cultures occurred in 125 (59 per cent) of 212 necropsies. The greatest discrepancy was due to the presence of a negative blood culture in 73 (34 per cent) of the individuals; 65 of these were in the clinical cultures and only 8 at postmortem. The most important factor responsible for the negative clinical cultures was the time intervening between the last clinical culture and the postmortem examination. Contamination accounted for the difference in 22 (10 per cent) of the necropsies. Mixed infections were present in 39 necropsies. In 65 postmortems in which both the clinical and postmortem cultures were negative, bacteria were isolated and demonstrated in the lesions found in the organs in 57 necropsies. Administration of specific immune sera, the phenomenon of bacterial dissociation and errors in technique of culture played a definite but minor rôle in some of these discrepancies.

EASTERN PENNSYLVANIA CHAPTER

PHILADELPHIA COUNTY MEDICAL SOCIETY BUILDING, NOVEMBER 24, 1936

IMMUNITY RESPONSE IN GUINEA-PIGS TO ALUM TOXOID. *W. P. Knerr, G. A. Hottle and F. L. Rights*, National Drug Co., Swiftwater, Pa.

In the purification of diphtheria toxoid with alum it was found that more than 1.0 per cent alum was retained, producing a greater antigenic response, if the pH was raised immediately after the addition of the alum to 7.8. In the antigenic tests in guinea-pigs injected subcutaneously with 1.0 cc. of purified toxoid, the highest titer was reached within four weeks, while six- and nine-week bleedings were each successively lower. Guinea-pigs bled out had a slightly higher titer than those given only trial bleedings.

HEMOLYSIS TEST FOR GAS GANGRENE TOXIN AND ANTITOXIN. *G. A. Hottle and C. Okono*, National Drug Co., Swiftwater, Pa.

Results agreeing within 15 to 30 per cent were obtained by the hemolysin test and the mouse intravenous test, when titrations were carried out on sera of horses immunized with *Clostridium welchii* (Perfringens) and *Clostridium oedematis-maligni* (Vibrion Septique) toxins. The hemolysin test has been used successfully in detecting amounts of antitoxin as small as 0.01 unit.

Observations on the Protection Test for Anti-meningococcic Serum. *J. Fertig*, National Drug Co., Swiftwater, Pa.

The results of mouse-protection tests on anti-meningococcic serum were reported. A comparison of unconcentrated with concentrated serum appeared to indicate that the concentrated preparation afforded approximately five times the protection afforded by the unaltered serum. Two commercial preparations appeared to possess very nearly the same protective powers. This was approximately five times the protection afforded by the National Institute of Health control serum. All tests were run with a type I culture.

Meningococcic Meningitis in an Eight-day-old Infant. *J. L. Ingham and F O. Zillessen*, Easton Hospital, Easton, Pa.

The child was a difficult breech delivery. From the time of birth until death both right extremities were cyanotic and the superimposed symptoms of meningococcic infection were difficult to distinguish from those of possible birth injury. The first two days of life were those of the normal newborn. On the third day the infant ran a fever of 101°F. and nursed poorly. From this time until death on the eighth day the weight dropped from 8 pounds 14 ounces to 8 pounds. The fever never exceeded 102.6°F.

Suspicion of central nervous system infection was aroused by an intense jaundice on the fifth day of life. A spinal tap revealed a yellow spinal fluid of increased viscosity under a pressure of 8 mm. of mercury. Microscopic examination showed many pus cells and the meningococcus. Ten cubic centimeters of anti-meningococcic serum were given daily, intramuscularly, without any marked effect. The child died on the eighth day of meningococcic meningitis. Clinical findings were confirmed by autopsy.

Cholecystitis Caused by Hemophilus influenzae. *S. E. Weintraub and F. O. Zillessen*, Easton Hospital, Easton, Pa.

The following is a summary of an acute cholecystitis caused by *Hemophilus influenzae:* (1) A history of an upper respiratory infection associated with headache, occasional chills and fever two weeks before the onset of the gall bladder symptomatology; (2) a marked leukopenia which, instead of responding to so-called specific treatment, became worse; (3) a drop of the white blood count to zero, and (4) a secondary reaction by the body before death as indicated by the rise of the white blood corpuscles to 4500.

Coexisting Typhoid and Paratyphoid "B" Infection. *J. Kincov and F. O. Zillessen*, Easton Hospital, Easton, Pa.

The patient on admission to the hospital had a history of five weeks duration of fever and diarrhea. The Widal test was positive in all dilutions for typhoid and paratyphoid "B" bacilli. Repeated cultures of the stools showed the constant occurrence of both organisms. The patient suffered a recrudescence on the 20th day of hospitalization, which lasted for 8 days, although there was no recurrence of the diarrhea. During this period the stools were positive for both organisms. The patient was discharged on the 41st day of hospitalization after three successive negative stools for typhoid and paratyphoid "B" bacilli. The case is presented because of the unusual occurrence of such dual infection.

Clostridium welchii Infection in the Dog Treated Successfully

WITH GAS GANGRENE ANTITOXIN. *A. F. Millar, W. B. Rawlings and W. G. Love*, Deal, New Jersey.

Gas gangrene, with its clinical symptoms, manifestations, and recovery has been observed in the dog. The history, symptomatology, pathology and treatment for this condition is similar to that in man, as written by the leading medical practitioners who have reported their findings. A complete laboratory examination, with confirmation, is a valuable aid in the making of an early diagnosis so that the proper treatment for this infection may be carried out.

UNCONCENTRATED VERSUS CONCENTRATED ANTITOXIN IN THE TREATMENT OF GAS GANGRENE. *F. O. Zillessen*, Easton Hospital, Easton, Pa.

A series of 20 cases, (out of 23,000 admissions over a 5-year period, an incidence of 0.087 per cent), were presented to show the value of concentrated antitoxin over unconcentrated antitoxin in anaerobic gas bacillus infection. The 20 cases were divided into three groups; two cases received no serum, 6 cases received unconcentrated serum and 12 cases received concentrated serum. A study of the units of antitoxin given in the various groups revealed that an average of 27,980 units per case were administered to those receiving the unconcentrated serum and an average of 169,451 units per case to those receiving the concentrated serum. Out of seven cases given prophylaxis, six recovered. The mortality for the entire series was 40 per cent. In the series receiving the unconcentrated serum, the mortality was 66 per cent, whereas the series receiving the concentrated serum showed a mortality of 16 per cent, which compares favorably with the lowest mortality quoted by Millar, namely 19 per cent.

Clinical and x-ray photographs were shown of the various cases presented, demonstrating the type of injury frequently seen, the presence of gas in the tissues and the typical gangrenous process in all anaerobic gas infections. A brief historical development of the use of protective sera was presented.

THE TEACHING OF BACTERIOLOGY TO PRE-MEDICAL STUDENTS. *W. R. Hunt*, Jenk's Biological Laboratory, Lafayette College, Easton, Pa.

The purpose of the paper is to give reasons why, the author believes, and why Lafayette College pre-medical students believe, that a general course in bacteriology is of value as a foundation for work in medical school. A college course covering a whole year must be broad in its scope, stressing basic technic and the fundamental theories and principles underlying its applications in industry, art, sanitary engineering, agriculture, dairying, chemistry and medicine. A general course, like this, does not interfere too seriously with the specialization met with in medical school.

NORTH CENTRAL BRANCH

UNIVERSITY OF MINNESOTA, NOVEMBER 13–14, 1936

SEROLOGY OF BACTERIAL SPORES APART FROM VEGETATIVE CELLS. *Elizabeth McCoy and Elizabeth Krauskopf*, Unisity of Wisconsin.

Antisera were prepared against the vegetative cells of *Bacillus niger* and against the spores of the same organism (treated with KOH to dissolve vegeta-

tive remnants). Cross agglutination reactions between the two antigens indicated close serological relationship between spores and vegetative cells. Absorption experiments revealed the presence of an H (flagellar) factor in spores, demonstrable both *in vivo* and *in vitro*. Comparisons were made of the reactions of alkali-treated as compared with untreated spore suspensions as test antigen. The treatment reduces the *in vitro* reactivity of the spores, especially their ability to absorb agglutinins, and destroys the ability of the H factor to agglutinate *in vitro*.

Dissociation in Monilia. *Elizabeth Pinkerton*, University of Nebraska, Omaha, Nebraska.

Fifty strains of *Monilia* secured from various pathological conditions in the human body have been studied regarding comparative morphology. On the basis of giant-colony formation, six types were recognized which seemed to agree fairly well in other characters. Representatives of the four main types were carried through several generations by simply making loop transfers. Results indicated a tendency to noticeable variation in giant-colony characteristics in most cases, although a few stable strains were encountered. In one variable strain followed through four generations, all of the progeny were segregated into four sub-types of which there was one main group around which the others varied with a normal curve distribution. In the same strain, forty-eight-hour pour-plate colonies were examined for S, R and r varieties. R varieties were infrequent and stable; S varieties were common and unstable; and r varieties were intermediate in both respects. S, R and r varieties were secured from single giant colonies in some cases.

Occurrence of Monilias in Tuberculosis Sputum. *Virginia M. Schwarting*, Glen Lake Sanatorium and the University of Minnesota, Minneapolis.

Monilias were isolated in 19.6 per cent of the sputums from tuberculous patients in a series of 500 cultures.

Of the total number of strains isolated, 65.3 per cent were from patients with far-advanced lesions, 24 per cent from moderately advanced and 10 per cent from patients with minimal lesions. In a series of 100 up-patients, only 7 per cent of the patients had Monilias in sputum. It is evident that the amount of tuberculosis in the lungs has some influence upon the incidence of Monilias in the sputum, although it is not clear in this study whether this influence is due to increased opportunities for growth offered by the linings of cavities and inflamed bronchial tubes of the far-advanced patient, or to the lowered resistance of such a patient.

Less than half the strains in a series of twenty-three studied, about 43.4 per cent, were pathogenic for mice, which does not necessarily indicate pathogenicity for man.

Studies on Yeast-like Organisms Isolated from the Mouths and Throats of Normal Persons. *Ramona L. Todd*, Minnesota Department of Health, Minneapolis.

A survey of the normal mycological flora of the mouth and throat was made to determine the incidence of yeast-like organisms and especially to note the presence of *Monilia albicans*. It was found that 14.7 per cent of 1,000 normal individuals harbored yeast-like fungi and in 14 per cent *Monilia albicans* was present. In 7 per cent, *Monilia* was present in both mouth and throat, in 3.1 per cent in the mouth only, and 3.9 per cent in the throat

only. There was a higher incidence in females: 18.2 per cent of 527 females and 9.3 per cent of 473 males yielded the organism.

Tests were made with human sera to determine whether agglutinins for *Monilia albicans* were present. Of sera from 1,150 normal persons, 259, or 22.5 per cent, agglutinated *Monilia albicans*, but only 35 reactions were of a titre of 1:160 or above. Results showed that agglutinins for *Monilia* were present in the sera of 30.4 per cent of 533 females and 15.7 per cent of 617 males. There seems to be some relationship between a high titre of agglutinins in the serum and the presence of *Monilia albicans* in the mouth and throat of an individual.

THE Vi ANTIGEN OF EBERTHELLA TYPHOSA. *L. A. Weed and W. J. Moore*, Department of Hygiene, Preventive Medicine and Bacteriology.

Cultures of Type 2 and Watson strains of *Eberthella typhosa* were compared serologically with O-901 and H-901 strains received from various laboratories. The Vi strains, (Type 2 and Watson) were "O" agglutinable and remained so even after extensive cultivation on guinea pig serum, after serial passage through mice, and after long-continued cultivation on soft ascitic agar. Virulence tests on white mice showed no appreciable difference between the strains supposed to contain Vi antigen and those devoid of it. We have been unable to correlate virulence with "O" inagglutinability of organisms freshly isolated from cases of typhoid fever.

AN AUTOCHTHONOUS BACTERIAL FLORA IN GREAT SALT LAKE. *W. Whitney Smith,*[1] University of Utah and *Claude E. ZoBell*, Scripps Institution of Oceanography, University of California.

During 1935 the Great Salt Lake reached the greatest density in its history. It was far saltier than the ocean having in December a salt content of 336 grams per liter. In this year large amounts of both NaCl and $Na_2SO_4 \cdot 10H_2O$ were precipitated out of the water.

Popular literature and earlier scientific reports to the contrary this lake contains a variety of small organisms including algae, protozoa, and bacteria. Most of the viable bacteria in the lake are indigenous as evidenced by the relatively even distribution of bacteria in different places in the lake including those remote from terrigenous contamination despite the fact that the water is highly bactericidal or bacteriostatic for bacteria from the ocean, freshwater, soil sewage and other sources.

Moreover, the direct microscopic observation of chemically clean and sterile glass slides which had been aseptically submerged in the lake according to the Henrici technic revealed the presence of individual bacteria and micro-colonies which were attached to the slides tenaciously enough to resist washing and staining without fixation.

Further evidence of an autochthonous bacterial flora is the preponderance of obligate halophilic bacteria as compared to others. From data concerning these the following approximation may be made. Fifty per cent of the lake bacteria require over 7 per cent salt to grow. Ninety-six per cent can not grow in the absence of salt.

PASSIVE IMMUNITY TO DISTEMPER. *R. G. Green*, Department of Bacteriology, University of Minnesota.

Tests with canine distemper dog

[1] Now of University of Wisconsin, Madison.

antiserum on foxes and ferrets have shown the heterologous use to have comparatively little effect in establishing passive immunity against a proven distemper virus adapted to the animal species tested. Similar results were obtained with two standard commercial dog serums and with several experimental dog serums.

THE AEROBIC AND ANAEROBIC DISSIMILATION OF THE PROPIONIC ACID BACTERIA. *H. G. Wood and C. H. Werkman*, Department of Bacteriology, Iowa State College, Ames.

The aerobic and anaerobic dissimilation of lactic and pyruvic acids, glucose and glycerol in yeast-extract medium has been compared by quantitative determination of the products as well as oxygen consumed, using the apparatus described by Wood, Erb and Werkman (Iowa State College, J. Sci., **10**, 295 (1936)). On the basis of 100 mM of fermented material the O_2 utilized varied from 113 to 212 mM. The effect of oxygen consumption was to decrease total volatile acid and practically eliminate formation of propionic acid. Pyruvic acid occurred as an end product and formation of CO_2 was greatly in excess of the mM acetic acid.

The aerobic mechanism of acetic acid formation is assumed to be the same as in anaerobic fermentation, i.e., from intermediate pyruvic acid. The increase in CO_2 probably originates by oxidation of acetic acid to succinic acid and its decarboxylation to propionic acid which is in turn oxidized with formation of CO_2. The oxidation of acetic and propionic acid with liberation of CO_2 has been demonstrated by experiments with cell suspensions employing the Barcroft-Warburg respirometer. Occurrence of the Thunberg-Wieland series in the breakdown of succinic acid is not supported since fumaric and malic acids are oxidized very slowly.

METABOLIC STUDIES ON THE PROPIONIC ACID BACTERIA. *Edgar L. Piret and Claude Fromageot*, University of Minnesota, and University of Lyon, France.

The propionic acid bacteria have been regarded as requiring for their development complex organic nitrogen. However, Laroux and Fromageot observed that *Propionibacterium* 11 is capable of utilizing the nitrogen of ammonium acetate in the presence of polenta extract. In this work, fermentations were followed with *Propionibacterium* 11, 12, 52, 56 and *Propionibacterium shermanii* (according to Hitchner's classification) in media containing glucose or lactic or pyruvic acid, ammonium acetate, and polenta extract as activator. The growths were found to depend both on the organism and on the carbohydrate. In the absence of polenta extract, no growth took place; in the absence of ammonium acetate, the growths were light, and corresponded to the slight amounts of nitrogen carried by the polenta extract. Other ammonium salts and amino acids were not utilized as easily as ammonium acetate. The results indicate that the propionic acid bacteria and possibly other fastidious organisms do not absolutely need, for their development, complex organic nitrogen but rather need the presence of another factor which is usually brought in by the medium itself. This factor would permit them to carry on synthesis starting from the ammoniacal nitrogen which comes from a previous decomposition of the amino acids of the medium.

THE UTILIZATION OF GLUCURONIC ACID, GALACTURONIC ACID, AND AN

Aldobionic Acid by Certain Bacteria and Yeasts. *Frithjof Setter*, University of Wisconsin.

The abilities of a wide variety of different bacteria and yeasts including rhizobia, lactobacilli, propionibacteria, clostridia, and a number of the common aerobic organisms, to utilize the uronic acids, galacturonic and glucuronic, and an aldobionic acid, were tested.

It was found that the rhizobia were capable of utilizing the uronic acids but not the aldobionic acid, while the yeasts did not attack any of the acids. Six of the eight butyric anaerobes tested were capable of utilizing the uronic acids while none of them were able to utilize the aldobionic acid.

One of the lactic acid organisms was capable of fermenting both the uronic acids and the aldobionic, while negative results were obtained with the other two lactic acid bacteria and the propionic acid bacteria which were tested.

Most of the common aerobes were able to utilize the uronic acids but not the aldobionic acid.

Evidence of a Rotational Growth Factor in Bacillus Mycoides. *James L. Roberts*, University of Wisconsin.

Within recent years, the question of spirality in plant and animal cells has received widespread attention. While little progress has been realized in the ultimate explanation of the phenomenon, in general it is believed that spirality arises from the resolution of two growth factors, one longitudinal and one rotational.

Earlier writers have shown that spirally twisted filaments of *Bacillus mycoides* cells do occur, but these have apparently not been associated with rotational growth.

By attaching a filament of cells firmly within agar, leaving one end of the chain free to move within a liquid medium, it has been possible to produce great numbers of spirally twisting filaments of *B. mycoides*. The assumption is made that these spiral twists result from a tension produced by rotation of the long axis when, at some point, the chain of cells is so firmly attached that the only relief is to break or twist spirally.

A perfect correlation between the direction of filament twisting and the direction of colonial spirality has been demonstrated.

It is suggested that *B. mycoides* may exhibit longitudinal and rotational growth factors, and that the resolution of these two factors may determine the typical colonial spirality of the organism.

The Effect of Metabolites on Growth as a Differential Character in the Colon Group. *M. J. Powers and Max Levine*, Iowa State College.

A Comparative Study of the Germicidal Activity of Certain Phenolic and Mercurial Compounds. *C. S. McCleskey and Edith L. Swingle.*

Various solutions and mixtures of *sec.* amyltricresol and O-hydroxyphenylmercuric chloride were compared with several widely used proprietary preparations. Phenol coefficients (F.D.A. method) were determined using *Eberthella typhosa* at 20°C. and *Staphylococcus aureus* at 20°C. and at 37°C. Germicidal tests in the presence of blood plasma were carried out with *Eberthella typhosa*, *Staphylococcus aureus*, *Streptococcus viridans*, *Bacillus metiens*, and *Clostridium welchii*. For the study of bacteriostatic effects in

blood plasma and in broth *Staphylococcus aureus* and *Bacillus metiens* were employed.

The phenol coefficient of the tincture of *sec*-amyltricresol was found to be about 17 times that of the same compound in 2 per cent soap solution, when *Eberthella typhosa* was employed as the test organism. On the other hand, when *Staphylococcus aureus* (37°C.) was employed, the soap solution of *sec*-amyltricresol was more than twice as effective as the tincture.

The *sec*-amyltricresol, in 2 per cent soap solution, was found to possess definite selective action against *Staphylococcus aureus*. At 20°C., it gave a phenol coefficient of 1.8 with *Eberthella typhosa*, whereas for *Staphylococcus aureus* it was 50, or about 28 times as great.

In the presence of blood plasma Mercresin (a mixture of *sec*-amyltricresol and O-hydroxyphenylmercuric chloride) was found to be more effective against *Streptococcus viridans* than any of the other compounds tested. The *sec*-amyltricresol preparations were uniformly more effective against the gram-positive cocci than O-hydroxyphenylmercuric chloride when plasma was present.

Against the spore-formers, *Bacillus metiens* and *Clostridium welchii*, none of the substances tested were particularly effective in the concentrations employed.

NODULATION OF SOYBEANS BY EFFECTIVE AND INEFFECTIVE STRAINS OF *Rhizobium japonicum*. *E. W. Ruf and W. B. Sarles*, Department of Agricultural Bacteriology, University of Wisconsin, Madison.

The number, weight, volume and distribution of nodules on the roots of soybeans grown in sterile sand, under greenhouse conditions, from seed inoculated with effective strains of *Rhizobium japonicum* was compared with the nodulation of soybeans grown from seed that had been inoculated with an ineffective strain. The relative effectiveness of the strains used was determined by comparing the total nitrogen content of the soybean plants grown from inoculated seed with that of plants grown from uninoculated seed.

Experiments performed in both fall and spring showed that effective strains produced relatively few, large nodules that were located on or in the immediate vicinity of the tap root near the surface of the soil. The ineffective strain produced many small nodules that were scattered over the entire root system. The number, weight and volume of nodules produced by the ineffective strain was greater than in the case of the effective strains.

When the roots of soybeans grown in the fall experiment were allowed to remain in the pots of sand throughout the winter, and were broken up with a sterile spatula the following spring, roots of soybeans grown from uninoculated seed that was planted in these pots were found to exhibit the same type of nodulation that was found on plants grown from inoculated seed. The ability of the various strains to benefit the host plant was not altered greatly by their life in the nodules and sand throughout the winter months.

FURTHER STUDIES ON THE INFLUENCE OF THE HOST PLANT UPON THE EFFECTIVENESS OF THE RHIZOBIA. *J. C. Burton, V. S. Bond, and W. W. Umbreit*, University of Wisconsin.

Studies on the ability of several strains of *Rhizobium meliloti* to fix atmospheric nitrogen in symbiosis with several closely related species as well as varieties of sweet clover and alfalfa have indicated that there is a wide

variance in their effectiveness. This variation was shown to be due not only to the strain of rhizobium used but also to the host plant. It has been reported previously that the genus of the host plant influenced the effectiveness of the strain; i.e., a given strain may be judged effective on alfalfa (*Medicago*) but ineffective on sweet clover (*Melilotus*). The studies here reported have shown that the differing ability to fix nitrogen extends not only to the genus of plant, but to species and to varieties within a species as well.

Biological Treatment of Cannery Waste. *H. Orin Halvorson*, University of Minnesota.

An investigation was made of the possibility of treating cannery waste by mixing it with domestic waste and dosing it on a trickling filter designed for the treatment of domestic waste alone. The particular filter used had been designed for a dosage of about 2 million gallons per acre per day. The volume of cannery waste was sufficient to increase the dosage to 4 million gallons per acre per day. Before mixing, the cannery waste was put through a fine screen. The reduction in B. O. D. with domestic sewage alone was about 90 per cent; with the added cannery waste, the B. O. D. reduction was about 85 per cent. Results show that cannery waste can be successfully handled by a trickling filter used for the treatment of domestic sewage.

CELL SIZE AND METABOLIC ACTIVITY AT VARIOUS PHASES OF THE BACTERIAL CULTURE CYCLE[1]

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Received for publication, May 26, 1936

OBJECTIVES OF PRESENT STUDY

Experimental studies on the morphology and physiology of unicellular organisms have provided convincing evidence that the cells of early generations developing in a fresh, favorable medium are quite different from those of generations developing after the period of maximum multiplication. That bacteria during the lag or early logarithmic growth phases are less resistant than cells of subsequent population periods to such inimical agents as heat, cold, 2 per cent NaCl, 0.5 per cent phenol and dilute crystal violet has been demonstrated by Schultz and Ritz (1910), Reichenbach (1911), Sherman and Albus (1923, 1924), Sherman and Cameron (1934), Stark and Stark (1929), Heiberg (1932) and Salter (1919). Research in the field of quantitative metabolism indicates that the onset of the period of maximum reproduction is accompanied by a marked increase in metabolic activity as measured by such indices as heat production (Bayne-Jones and Rhees, 1928; Schmidt and Bayne-Jones, 1933), oxygen consumption (Eaton, 1931; Gerard and Falk, 1931; Martin, 1932), carbon dioxide and ammonia production (Cutler and Crump, 1929; Meiklejohn, 1930; Walker and Winslow, 1932; Walker, Winslow, Huntington and Mooney, 1934; Mooney and Winslow, 1935). Metabolic activity apparently declines before multiplication ceases to take place at the maximal rate. These phenomena have come to be regarded as manifestations of a

[1] Based on a thesis presented by E. H. in partial fulfilment of the requirements for the Doctorate in Philosophy of Yale University.

period of physiological youth comparable to that exhibited in the life cycle of a multicellular organism.

Measurements of bacterial cells indicate that individuals of a large number of species increase in size during the early hours of culture growth, but soon decrease to approximately the original level. Thirty-five of the thirty-seven species studied by Clark and Ruehl (1919) showed such a size cycle. For the majority of these species a maximum was reported at four to six hours after transplantation, when the cultures were in the logarithmic growth phase.

Wilson (1926) observed that suspensions of four-hour and twenty-six-hour cultures of *Salmonella aertrycke* containing the same number of organisms were dissimilar in opacity and concluded that the cell volume of the younger organisms was five times as great as that of the twenty-six-hour cells.

Data on cell size of *Bacillus megatherium* and *Escherichia coli*, as well as of other species, have been accumulated by Henrici (1928) from the projected images or photomicrographs of organisms treated with Congo red and HCl. Cells of *Escherichia coli* grown on agar increased in length from 1.5 to 4 micra in the first three hours after inoculation and subsequently declined in size. Jensen (1928) reported an increase in size of the cells of *Escherichia coli* during the first hour of growth on agar as well as in broth and a decrease after the third hour of culture development.

Bayne-Jones and Adolph (1932) obtained consecutive records of changes in the size and rate of growth of individual cells of *Escherichia coli* and *Bacillus megatherium* cultivated on agar, by means of motion photomicrography. With *E. coli* it was found that the mean rate of growth in volume of individual cells reached a maximum sixty minutes after inoculation of the medium and then declined rapidly, whereas the mean rate of reproduction did not reach a peak until two hours after inoculation. Maximum cell volume (prior to fission) was greatest at ninety minutes and thereafter diminished progressively, the last cells observed being only one-fifth the size of the first cells.

An investigation of the effect of agitation on bacterial growth

was made by Mudge and Smith (1933). Their data indicate that, although agitated and unagitated cultures of *Escherichia coli* increase to a similar maximum cell size in the early logarithmic growth phase, cells of the agitated cultures are only one-third as long as cells of unagitated cultures after the close of this period.

Using a photo-electric cell in measuring the opacity of cultures of *Salmonella gallinarum*, Alper and Sterne (1933) found that the organisms increased in size for three hours following inoculation and then decreased until the seventh hour when the cells were similar in size to those from twenty-four-hour cultures.

In considering these various observations it is obvious that the increased metabolic activity characteristic of the phase of physiological youth might be accounted for by the larger size of the individual cells at this period, since metabolic activity has ordinarily been computed on a cell-per-hour basis. Walker, Winslow, Huntington and Mooney (1934) made a preliminary attempt to analyze this problem by using data for metabolic activity of *E. coli* obtained in this laboratory, on heat production reported by Bayne-Jones and Rhees (1928) and on oxygen consumption reported by Martin (1932) in comparison with cell measurements reported by Bayne-Jones and Sandholtzer (1933) for the same organism. They concluded that size changes could only in part account for increased metabolic activity. To date, however, the only report citing direct simultaneous observations of size and metabolic activity is that of Martin (1932) which dealt with the oxygen consumption of *Escherichia coli* per cell per minute during the lag and logarithmic growth phases. The rate of oxygen consumption reached a maximum near the end of the lag phase and then declined gradually. Cell measurements showed a rise and subsequent fall in cell size with a two-fold increase in surface area of the largest over the initial cells. The absolute point of maximum surface area varied with separate experiments, but in relation to the growth curve it also coincided in almost every instance with the end of the lag phase.

The problem seemed of sufficient importance to warrant a more intensive simultaneous study of cell size and metabolic

activity in several different types of organisms and in several media. The primary purpose of the investigation was to determine how nearly changes in cell size parallel changes in the hourly rates per cell of carbon dioxide production and whether computations of metabolic activity per unit volume of bacterial substance vary significantly at different phases of population development, when size measurements and data on metabolic activity are simultaneously determined. Observations on *Escherichia coli* were made to check previous data on carbon dioxide production and obtain figures for cell size under the cultural conditions employed in this laboratory. *Salmonella gallinarum* and *Salmonella pullorum* were also included in the study to discover whether these species, which differ in fermentative capacity from *E. coli*, show changes in cell size and metabolic activity similar to or distinct from those exhibited by the colon bacillus, in the presence and in the absence of carbohydrates.

METHODS

Samples for determinations of cell size were taken hourly from cultures growing at 37°C. in fluid media, continuously aerated with air freed from carbon dioxide and ammonia. Amounts of carbon dioxide produced, cell counts and rates of carbon dioxide production per cell per hour for the same cultures (which were used for a joint study) have been reported by Mooney and Winslow (1935).

Since the methods of determining carbon dioxide production and cell counts have been described in that report as well as by Walker and Winslow (1932), and Walker *et al.* (1934), it is sufficient to state that both the carbon dioxide in the medium and that carried off by aeration were measured for successive hourly periods and samples for cell counts and size measurements taken, from inoculation to the end of the logarithmic growth phase; a period of two or three hours between the twenty-second and twenty-eighth hour was also observed. Bacterial numbers were counted by the dilution and pour-plate method.

The experimental conditions of this study were such that it was necessary to make determinations of cell size on material

which had been fixed and stained. Consequently smears were prepared in the usual manner on glass slides which were then stored until measurements could be made. The apparatus used for measuring the bacteria consisted of a Spencer filar micrometer with a 10× ocular, a monocular microscope equipped with a mechanical stage and a Zeiss oil immersion, apochromatic objective magnifying 90×. We found a 10 per cent aqueous solution of Ziehl's carbol-fuchsin applied for three minutes the most satisfactory stain for this work since it was more effective than methylene blue or safranin in dyeing the cells during the later hours of culture growth.[2]

Measurements of two-, three-, and five-hour cells of *Escherichia coli* stained in this manner were compared with those of cells observed unstained in a drop of 1 per cent agar under a cover glass. The mean of 100 or more unstained cells was 4.2 by 0.9, 3.7 by 0.8, and 2.2 by 0.8 micra at two, three and five hours respectively. The corresponding figures for stained cells from the same cultures were 3.1 by 0.7, 2.7 by 0.7 and 1.6 by 0.65 micra, thus showing that the unstained organisms appeared 20 to 25 per cent larger than the stained forms. Although the processes of fixing and staining undoubtedly result in some cell shrinkage, it seems likely that the optical properties of the unstained preparation may give values in excess of the true dimensions—which perhaps lie between the two extremes. In any case, the employment throughout the work of one technique for fixing, staining and measuring should make evident any relative changes in cell size.

To determine the number of cell measurements necessary for obtaining a representative sample of the bacterial population at each observation point a statistical analysis was made of deviations obtained by counting and averaging various numbers of individual cells. This study indicated that a sample of twenty-five or thirty organisms is adequate for obtaining a reasonably

[2] The precipitation of Congo Red by peptone recorded by some workers has not been observed in this laboratory. The negative staining procedure of Benians might therefore be used and would perhaps give sizes closer to those of unstained cells.

representative mean. (See table 1 for a typical example of actual standard deviations.) Throughout our later work therefore thirty organisms from each culture have been measured at each observation point. The validity of this procedure has been demonstrated by the agreement between means when it has been necessary after a lapse of several months to measure a second or third group of thirty organisms from one slide.

TABLE 1

Mean cell volume of individual cultures of E. coli in peptone medium (cubic micra)

EXPERIMENT NUMBER	AGE (HOURS)									
	0	1	2	3	4	5	6	7	23	25
63	43	65	1 18	76	61	.36	32	.30	27	31
64	44	57	84	1 03	.60	.49	.24	29	24	24
65	.39	45	86	65	.70	46	31	24	22	.22
66	.44	64	92	92	.67	.42	35		25	28
67	.36	52	1 24	1 03	65	.58	.41	36	26	25
68	29	59	1 18	1 09	.88	.80	50	37	.30	.31
Mean	39	57	1.04	91	.69	.52	36	.31	26	27
Standard deviation	± 07	± 07	± 17	± 18	± 04	±.13	±.06	± 06	± 03	± 02

In computing mean cell volume the formula

$$V = \pi\left(\frac{\text{width}}{2}\right)^2 \times (\text{length minus width}) + \frac{4\pi}{3}\left(\frac{\text{width}}{2}\right)^3$$

was used, assuming that the average cell could be considered as a cylinder bounded on each end by a hemisphere.

It was essential that the size of cell aggregates rather than of individual cells be used in computing rates of carbon dioxide production per unit volume of bacterial substance because bacterial numbers were determined by plate counts. Therefore, all values given refer to the size of cell aggregates. Aggregate and individual cell length differed most widely with *Salmonella gallinarum*, the former being 12 to 15 per cent greater than the latter at three, four and five hours, while the mean aggregate cell length of *Escherichia coli* was only 5 per cent greater than the mean cell length of individual cells.

Rates of carbon dioxide production per unit volume of bacterial substance per hour were calculated by the Buchanan formula as used by Walker and Winslow (1932) for determining the fermentative capacity of a single cell. Values for reproductive rate per hour were computed by the usual formula.

Eight series of experiments were made as follows: *Escherichia coli* in 1 per cent peptone and in 1 per cent peptone with 0.5 per cent glucose; *Salmonella gallinarum* in 1 per cent peptone, 1 per cent peptone plus 0.5 per cent glucose, and 1 per cent peptone plus 0.5 per cent lactose; *Salmonella pullorum* in 1 per cent peptone, 1 per cent peptone plus 0.5 per cent glucose plus 0.5 per cent NaCl,[3] and 1 per cent peptone plus 0.5 per cent lactose. For each of these experimental conditions six duplicate cultures were observed, except for *S. gallinarum* in peptone-glucose of which seven cultures were observed and for *S. pullorum* in peptone of which only four cultures were included. Two duplicate cultures and one uninoculated control were kept under simultaneous observation on a given day in order to minimize potential variations in the rate of aeration, water-bath temperature density of the inoculum and other environmental factors. However, in spite of the fact that every effort was made to keep the experimental conditions constant and to repeat without alteration each essential manipulation, individual cultures of each series showed a considerable degree of variation. Therefore, cell counts and amounts of carbon dioxide produced in all six or seven duplicate experiments under each of the eight experimental conditions were separately averaged as by Mooney and Winslow and the resultant hypothetical mean values compared. In the same way values for mean cell size of each culture at each observation point have been averaged in the present study for computing cell volume of the mean culture and calculating hourly rates of carbon dioxide production per cubic micron of bacterial substance. Data for cell volume of the mean culture of *E. coli* in peptone water as well as of the six individual cultures on which this mean is based appear in table 1 and are shown

[3] Mooney and Winslow (1935) found that the addition of NaCl was essential for the growth of this organism under the conditions of the experiment.

graphically in figure 1. This group of experiments was selected at random for demonstrating variation in cell size of individual cultures from the mean. Judging by the original work sheets, the six duplicate experiments varied more under this condition than under any other. The variation observed may be in part a fault of the technique used for measuring cells (particularly since values for width are difficult to measure accurately), and in part a result of the relatively long time interval between observation points (which involves variation in the exact phase of the culture cycle observed). The influence of such variation

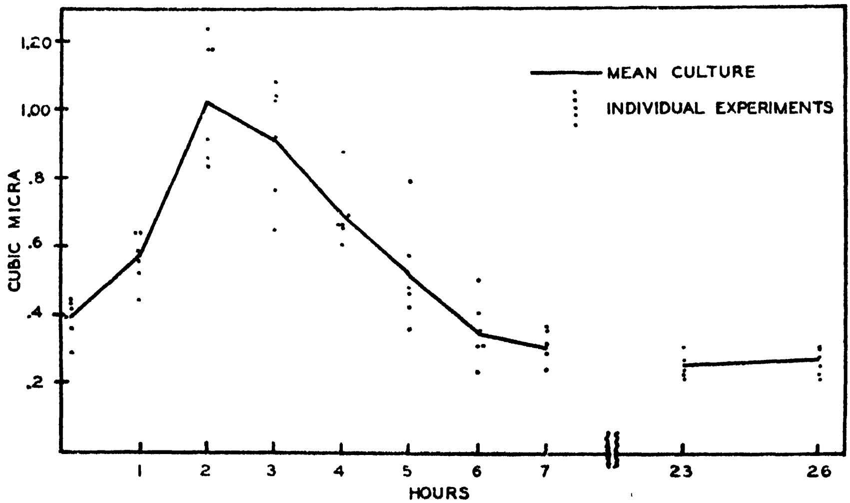

FIG. 1. CELL VOLUME OF E. COLI IN PEPTONE WATER BY HOURS

on cell numbers and cell size has, however, we believe been largely eliminated by averaging individual cultures and determining mean culture performance. Our results as presented are based on the actual measurement of over 13,000 individual cells.

EXPERIMENTAL RESULTS ON CELL SIZE

Our mean results for each organism and each medium are presented in tables 2 to 4. Curves of average cell volume appear in figure 2 where each panel contains the data for one species.

Comparison of these graphs and tables brings out four points of interest.

First, all the organisms in all the media exhibit the same general phenomena—an increase in cell volume followed by a decrease. The initial cell volumes vary only from 0.31 to 0.46 cubic micron and average 0.39 cubic micron. The final cell volumes (except in peptone-glucose) vary from 0.26 to 0.42 cubic micron, and average 0.34 cubic micron. Thus, we may consider the normal volume of all these organisms as between 0.3 and 0.4 cubic micron. At the maximum (excepting for *S. gallinarum* in peptone-lactose) the size varies from 0.76 to 1.28 cubic micra and averages 1.02 cubic micra, a remarkably close check considering that our hourly examinations could not always catch the cultures at the point of maximum size. Thus, in general the cell volume at the peak of physiological youth is three times that of the normal stable phase.

TABLE 2

Reproductive rate, cell volume and metabolic activity of E. coli

(Maximum value in heavy type)

TIME*	PEPTONE			PEPTONE-GLUCOSE		
	Metabolic activity†	Cell volume‡	Reproductive rate§	Metabolic activity†	Cell volume‡	Reproductive rate§
0		39			41	
1	116	57	02	78	77	− 13
2	**135**	**1 04**	40	**86**	**1 03**	1 04
3	90	91	92	79	.93	**1 41**
4	100	69	**1 17**	59	.91	64
5	95	52	.56	31	81	42
6	68	36	94	24	84	04
7	43	31	45	19	.89	18
23		.26			76	
25	8	27	− 60	7	75	05

* Interval after inoculation in hours.

† Milligrams $\times 10^{-11}$ of CO_2 per cubic micron per hour for preceding hour.

‡ Mean cell volume in cubic micra at stated hour.

§ Reproductive rate during preceding hour. Computed from formula,

$$\frac{1}{t} \times ln \frac{b}{B}$$

where *B* and *b* equal initial and final numbers.

The first exception to this general rule is the very great increase of size for *S. gallinarum* in peptone-lactose. Its maximum volume is 1.81 cubic micra, or nearly six times the size of the stable phase. This phenomenon can be explained only in part by a tendency of this organism to form chains in this medium.

TABLE 3

Reproductive rate, cell volume and metabolic activity of S. gallinarum

(Maximum value in heavy type)

TIME*	PEPTONE			PEPTONE-GLUCOSE			PEPTONE-LACTOSE		
	Metabolic activity†	Cell volume‡	Reproductive rate§	Metabolic activity†	Cell volume‡	Reproductive rate§	Metabolic activity†	Cell volume‡	Reproductive rate§
0		31			.38			42	
1	11	35	— 04	82	.37	09	53	53	01
2	61	60	03	102	.78	24	70	1 07	— 25
3	113	**.98**	09	85	**1 28**	12	120	**1 81**	05
4	**149**	95	58	**132**	97	56	122	1 47	.11
5	76	85	**1 14**	117	83	1.11	**191**	1 37	69
6	55	.53	.92	61	.72	**1.28**	114	77	1 32
7	43	.51	49	42	64	.24	42	63	**1 45**
8	31	.44	.70	28	.69	.36	21	.53	53
9	25	44	.12	15	.69	08			
10	17	.42	.19	11	.70	07			
23		.41			.65			33	
25	12	.41	—.57	5	.64	—.29	19	32	— 56

* Interval after inoculation in hours.

† Milligrams $\times 10^{-11}$ of CO_2 per cubic micron per hour for preceding hour.

‡ Mean cell volume in cubic micra at stated hour.

§ Reproductive rate during preceding hour. Computed from formula,

$$\frac{1}{t} \times ln \frac{b}{B}$$

where B and b equal initial and final numbers.

Individual cells were also distinctly larger in peptone-lactose than in peptone.

The second exception to the usual course of the observed phenomena is the interesting fact that cells grown in peptone-glucose tend with all organisms to remain larger throughout the later hours of the population cycle than is the case in other

media. The phenomenon is most marked in the case of *E. coli* but comparing initial values in this medium we find for all three organisms an initial range between 0.35 and 0.41, and a final range between 0.48 and 0.75 in the presence of glucose.

TABLE 4

Reproductive rate, cell volume and metabolic activity of S. pullorum

(Maximum value in heavy type)

TIME*	PEPTONE			PEPTONE-GLUCOSE			PEPTONE-LACTOSE		
	Metabolic activity†	Cell volume‡	Reproductive rate§	Metabolic activity†	Cell volume‡	Reproductive rate§	Metabolic activity†	Cell volume‡	Reproductive rate§
0		46			.35			.41	
1	170	46	16	17	.34	− 30	96	41	.30
2	**216**	73	.07	70	.56	−.17	59	60	17
3	178	96	05	52	.73	.27	**110**	**93**	.18
4	128	**1 13**	42	133	**.76**	.22	85	81	.52
5	108	81	55	**178**	56	88	67	71	.53
6	115	90	32	171	56	.59	78	.58	56
7	114	78	76	119	.43	**1 52**	71	55	**.78**
8	85	82	85	61	42	64	44	.46	47
9	40	63	**1 08**	36	.56	.16			
10	37	60	.30						
23		42			.48			30	
25	15	42	07	18	.48	− 04	13	.34	− 21

* Interval after inoculation in hours.

† Milligrams $\times 10^{-11}$ of CO_2 per cubic micron per hour for preceding hour.

‡ Mean cell volume in cubic micra at stated hour.

§ Reproductive rate during preceding hour. Computed from formula,

$$\frac{1}{t} \times ln \frac{b}{B}$$

where B and b equal initial and final numbers.

A clue to the problem of why cells remain larger in peptone-glucose than in peptone might be sought in accordance with observations by Stearn and Stearn (1933) in differences in the hydrogen-ion concentration of the two types of cultures. At the first hour the pH of all cultures lay between 6.7 and 7.0; as growth progressed the pH of the peptone cultures rose to values of 7.5 to 7.8 at seven hours, while the pH of peptone-glucose

cultures fell to 5.4 to 5.9. On the second day of culture growth the differences in pH were even greater, with 8.0 the value for peptone cultures and 4.6, 4.7 and 5.0 the respective values for *E. coli*, *S. gallinarum* and *S. pullorum* in peptone-glucose.

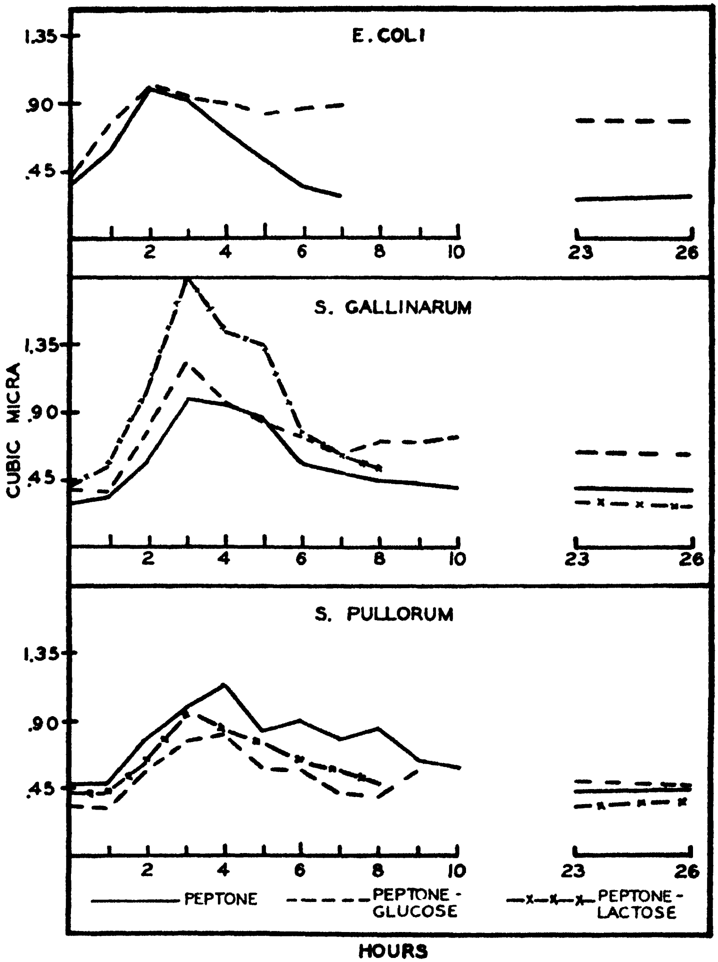

FIG. 2. AVERAGE CELL VOLUME OF THREE ORGANISMS BY HOURS

On the other hand, the increased nutrient concentration resulting from the addition of 0.5 per cent glucose to the medium might have been held responsible for alterations in mean cell

size. In a medium containing only 1 per cent peptone the decrease in cell size which occurred during and after the logarithmic growth period might reflect cellular adaptation to an environment containing a diminishing food supply. In such an environment the foodstuff would be more readily available if the bacterial culture were made up of small cells than if the culture were composed of larger cells, since in the latter case the surface-volume ratio is less. In a peptone medium to which 0.5 per cent glucose had been added the available food supply might have been such that the essential balance of assimilation of nutrients and excretion of metabolites could be maintained while cell size remained at a higher level than in peptone. Cell size figures given by Henrici show that *B. megatherium* did not increase to as great a maximum length in various dilutions of nutrient agar as in full strength nutrient agar, although the organisms did in every instance rise to a peak and then decrease to lengths less than those of the parent cells.

The enormous increase in cell size of *S.gallinarum* in peptone-lactose cannot, of course, be accounted for on either supposition since the sugar is not fermented and the medium remains alkaline.

Finally, it is of interest to note that the period of maximum cell size varies with the species of organism, coinciding with the second hour for *E. coli*, coming in the third hour for *S. gallinarum* and in the third or fourth hour for *S. pullorum*. It precedes the period of maximum reproductive rate by from one to five hours.

The point at which maximum cell volume occurred in relation to the growth curve of all the cultures reported in this study differed to some extent from that at which Henrici (1928) observed his longest cells. He concluded that *E. coli* cells reached maximum size during the logarithmic phase whereas we have found that cells were largest just before this period. However, Henrici reported no data on cell size between inoculation and the end of the third hour. During this period cell length very probably reached a higher value than that observed for cells three hours old. Other factors which may have been instrumental in yielding a different size-to-number relationship are the

composition of the medium, the density of the inoculum and lack of aeration. Since curves of cell lengths and cell numbers of *Bacillus megatherium* exhibited by Henrici show that the lag in cell length is shorter than the lag in reproduction our findings are essentially in agreement with his data, as well as with those of Martin (1932).

The bacterial cultures and the methods and intervals of observation of this study are not strictly comparable with those employed by Bayne-Jones and Adolph (1932) and Adolph and Bayne-Jones (1932) in measuring the growth in size of *E. coli* and *B. megatherium*. Although there was no way of determining the degree of maturity or the rate of growth of individual cells by the technique used in the present study, the fact remains that for each culture, maximum mean cell volume preceded the beginning of the maximal rate of reproduction and that cell size diminished during the logarithmic period of increase in numbers. In these respects our findings are in accord with those of Bayne-Jones and Adolph who observed that the maximum adult cell volume of *E. coli* was attained by the original cells inoculated on fresh agar and that, although the maximal growth rate of individual cells occurred one hour after inoculation, fission did not take place at the maximal rate until two hours after inoculation. (With *B. megatherium* the figures of Adolph and Bayne-Jones suggest that maximum cell volume appeared *after* the maximal rate of reproduction had been established.)

RATES OF CARBON DIOXIDE PRODUCTION ON A VOLUME BASIS

Data on rates of carbon dioxide production per cubic micron of bacterial substance per hour have been grouped by species in figure 3. Here again it is seen that, as growth in cell numbers and size vary at various phases of the culture cycle, so do rates of metabolic activity per unit volume of bacterial substance vary. All organisms in all media show a rise to a peak and a final fall to a point at, or below, the initial value. Thus, even when increased cell size is taken into consideration the late lag and early logarithmic growth periods are characterized by accelerated

metabolic activity. However, the excess in activity at these periods as compared with later phases is not as great when carbon

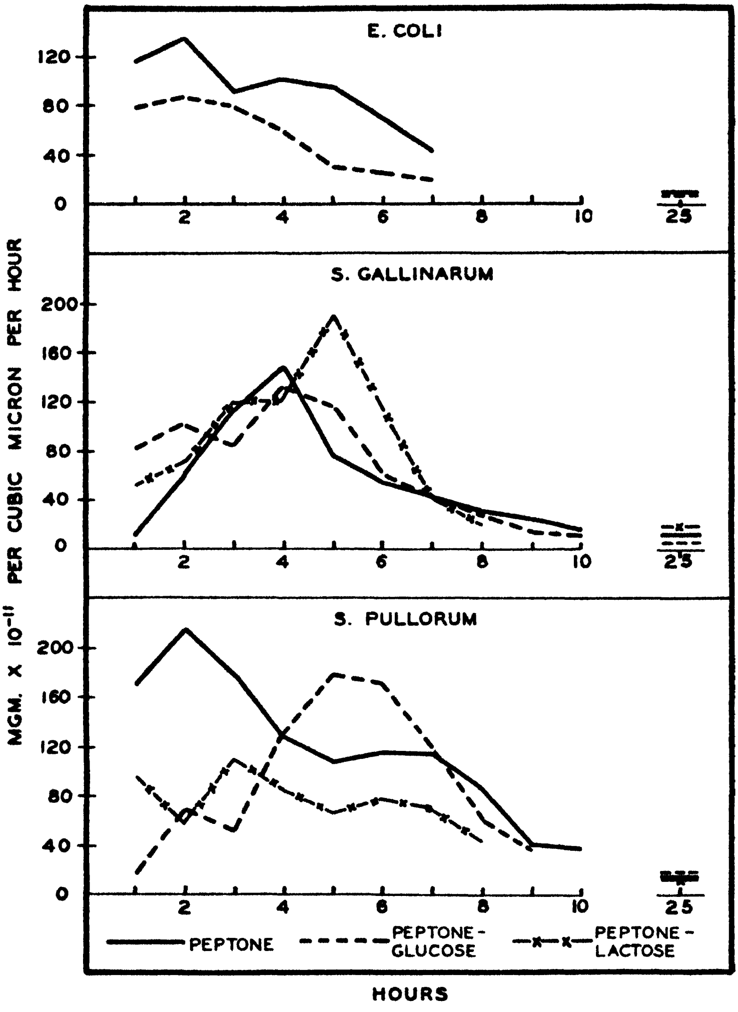

FIG. 3. HOURLY RATES OF CO_2 PRODUCTION PER CUBIC MICRON OF BACTERIAL SUBSTANCE OF THREE ORGANISMS BY HOURS

dioxide production is computed as a rate per unit mass of bacterial substance as when the product is calculated on a cell-per-hour basis.

Under the conditions of continuous aeration employed in these experiments, the presence of fermentable carbohydrates in the medium has no marked effect (with two exceptions which will be discussed later), upon metabolic activity as measured by yield of carbon dioxide. In the case of *E. coli* the amount of carbon dioxide produced in peptone-glucose per cubic micron was even less than that produced in peptone. With *S. gallinarum*, both maximal and final rates of carbon dioxide production in peptone-glucose were lower than in peptone although the sugar was actively attacked as demonstrated by the shift in pH from 7.0 to 5.3. With *S. pullorum* maximal yields were higher in peptone than in either peptone-glucose or peptone-lactose.

The failure of *E. coli* and *S. gallinarum* to liberate greater amounts of carbon dioxide per cell per hour in media containing available sugar has been reported in earlier studies made in this laboratory (Walker and Winslow, Mooney and Winslow). It was suggested that the apparently inconsistent effect of glucose in accelerating growth without increasing the rates per cell of carbon dioxide production would be reconciled by finding that cells were smaller in peptone-glucose than in peptone. Our measurements now show that such is not the case and that cells from peptone-glucose cultures are actually larger than cells from peptone cultures, thereby accentuating the difference between rates of carbon dioxide production by *E. coli* in the two media.[4]

Two exceptionally high rates of carbon dioxide production are indicated in figure 3, for *S. pullorum* in peptone (216 mgm. $\times$ 10^{-11} per cubic micron per hour) and for *S. gallinarum* in peptone-lactose (191 mgm. $\times$ 10^{-11}). For the first of these high rates we have no special explanation. The high rate for *S. gallinarum* in peptone-lactose may in part be explained by the fact that Dr. H. H. Walker in this laboratory has found plate counts of *S. gallinarum* in peptone-lactose to be abnormally low during the early hours in comparison with direct cell counts. This discrepancy between visible and viable cells was only in part the result

[4] A member of the JOURNAL editorial board has made the interesting suggestion that lower CO_2 yield and greater cell size in presence of fermentable carbohydrate might be related to increased formation of capsular carbohydrate material.

of relatively large numbers of chains of organisms. There was apparently some additional characteristic of lactose as it was used in these experiments which markedly reduced the ability of this organism to withstand dilution and plating on extract agar In view of this phenomenon, and of the additional observation that the experimental cultures increased in turbidity even though plate counts remained low it seems likely that the actual number of viable cells present was greater than that recorded by us.

In general, it is obvious that during the phase of positive growth acceleration the presence in the experimental cultures of viable cells which fail to develop on plates might tend to lower our estimate of bacterial numbers and volume of bacterial substance and thus to make our estimates of metabolic activity too high. Dr. Walker's studies of *E. coli* and *S. gallinarum* by direct microscopic counting methods indicate, however, that this error is not serious except in the case mentioned. The direct count would, of course, yield results considerably too high in the later phases of the culture cycle when cells are dying out. Therefore the plate count is the soundest basis for comparative studies.

The summary presented below indicates that in both peptone and peptone-glucose media *E. coli* is the least active and *S. pullorum* is the most active, metabolically, of the three species studied. In peptone-lactose, on the other hand, *S. gallinarum* seems to be more active than *S. pullorum*. This latter difference may, however, be due to the presence of viable but non-cultivable cells, to which reference has been made above.

Milligrams $\times$ 10^{-11} of CO_2 per cubic micron per hour

	PEPTONE	PEPTONE-GLUCOSE	PEPTONE-LACTOSE
Maximal:			
E. coli	135	86	
S. gallinarum	149	132	191
S. pullorum	216	178	110
Final:			
E. coli	8	7	
S. gallinarum	12	5	19
S. pullorum	15	18	13

From the standpoint of the general physiological characteristics of the culture cycle, it is of marked interest to consider the relative pace at which metabolic activity, cell volume and reproductive rate, increase. The periods at which maximum activity is attained in each of these respects is indicated below.

Period of maximum activity (hour after inoculation)

	SIZE OF CELLS	METABOLIC ACTIVITY	REPRODUCTIVE RATE
E. coli:			
Peptone	2	2	4
Peptone-glucose	2	2	3
S. gallinarum:			
Peptone	3	4	5
Peptone-glucose	3	4	6
Peptone-lactose	3	5	7
S. pullorum:			
Peptone	4	2	9
Peptone-glucose	4	5	7
Peptone-lactose	3	3	7

Cell size reaches a maximum first in four instances and metabolic activity once, while they are simultaneous in three cases. Cell multiplication is the last of the three characteristics to reach its peak in every instance.

There is a possible fallacy involved in this interpretation since cell size measured at a given hour obviously represents conditions at the end of that hour while the rates of cell multiplication and of metabolic activity recorded at the same time represents the mean of growth and metabolism during the preceding hour. The differences are, however, so marked as to leave no doubt of the fact that the increases of both cell volume and metabolic rate precede by a considerable interval increase in rate of cell division.

The relation between metabolic activity and cell size is less clear. The maximum of cell size, as noted above, is reached (except with *S. pullorum* in peptone) before, or simultaneously with, maximum metabolic activity. If, however, we assume that the metabolic activity and cell size after twenty-four hours

may be taken as a norm, it is evident that increase in metabolic activity is *initiated* long before increase in cell size. Taking the results at the end of the first hour we have the following ratios for cell size and metabolic activity, as compared with the corresponding data for the stable period on the second day.

Ratio of one-hour values to twenty-four-hour values

	E. COLI		S. GALLINARUM			S. PULLORUM		
	Peptone	Peptone-glucose	Peptone	Peptone-glucose	Peptone-lactose	Peptone	Peptone-glucose	Peptone-lactose
Cell volume	2	2	1	<1	1	1	1	1
Metabolic activity	14	11	1	16	3	11	1	7

In no case has the cell volume increased more than two-fold by the end of the first hour while in all but two instances the metabolic rate during the first hour is markedly above that for the stable period (three to fourteen-fold). In the two exceptional cases (*S. gallinarum* in peptone and *S. pullorum* in peptone-glucose) the cycle is delayed and the whole of the first hour is in the lag phase. For *S. gallinarum* in peptone the ratio of cell volume at the end of the second hour to that of the final phase was less than 2 and the corresponding ratio for metabolic activity was 5; for *S. pullorum* in peptone-glucose the second-hour ratios were 1 and 4, respectively. Clearly, metabolic activity begins to increase first, followed by cell volume and, finally, by reproductive rate.

Comparison of the three different organisms shows that *E. coli*, which exhibits the shortest lag in cell size, also reaches its maximum metabolic activity and its maximum reproductive rate most promptly. *S. gallinarum*, reaching its maximum cell size after *E. coli*, also attains maximum metabolic rate and maximum reproductive rate, more slowly than *E. coli*. *S. pullorum* attains maximum cell size and maximum reproductive rate last of the three organisms but shows (except in peptone-glucose) an earlier peak of metabolic activity than does *S. gallinarum*. For *Escherichia coli* in the two media, for *Salmonella gallinarum* in

all three media and for *Salmonella pullorum* in peptone-glucose, the first hour of the logarithmic growth period was characterized by maximal rates of carbon dioxide production. For *Salmonella pullorum* in peptone- and in peptone-lactose, maximum metabolic activity appeared before the period of logarithmic growth was even initiated.

SUMMARY OF CONCLUSIONS

1. Measurements of fixed and stained samples of continuously aerated, fluid cultures of *Escherichia coli*, *Salmonella gallinarum* and *Salmonella pullorum* indicate that mean cell volume increases during the lag period of culture development, reaching a maximum substantially before multiplication occurs at the maximal rate. During the logarithmic phase of rapid multiplication mean cell volume diminishes. The cells of the three organisms studied have a volume of about one-third of a cubic micron in their stable phase and increase to maximum sizes of about one cubic micron (in the case of *Salmonella gallinarum* in peptone-lactose to nearly two cubic micra).

2. When the amounts of carbon dioxide produced by these cultures are used to compute hourly rates of metabolic activity per cubic micron of bacterial substance, it appears that increased cell volume does not adequately account for the relatively greater amounts of carbon dioxide produced during the lag period and early part of the logarithmic growth phase. Although allowance for variations in cell volume eliminates a portion of the discrepancy between metabolic activity in the early and later hours of culture growth, the highest hourly rates of carbon dioxide production per cubic micron, which generally coincide with the first hour of logarithmic increase in cell numbers, vary from 86 to 216 mgm. $\times 10^{-11}$ of CO_2 per cubic micron per hour. They are from ten to twenty times greater than rates computed for later periods of negligible population change. This necessitates the conclusion that the cells appearing at the end of the lag period and the beginning of the phase of logarithmic reproduction are distinct in metabolic activity, as well as in size, from cells developing thereafter. Taken in conjunction with the observations of

earlier workers, the data reported in the present study appear to give additional support to the hypothesis that bacterial cultures exhibit a period of physiological youth closely resembling that of more highly differentiated multicellular organisms.

3. The three characteristics of physiological youth studied by us exhibit a definite and orderly relationship, metabolic activity increasing first, followed by cell volume and, in turn, by cell division rate. After the peak has been reached, cell volume and metabolic activity decrease rather rapidly while cell division rate continues high for several hours.

4. Each of the three species of bacteria exhibits a characteristic time cyle in all these relationships, *Escherichia coli* reaching its phase of physiological youth most rapidly, *Salmonella pullorum* least rapidly.

5. The presence of a fermentable sugar, glucose, seems to stimulate more rapid cell-division in the early phase of the culture cycle than is observed in plain peptone media with *Escherichia coli* and *Salmonella pullorum.* The stimulating effect of glucose is not, however, accompanied by any increase in yield of carbon dioxide per cubic micron of bacterial substance. In the presence of glucose both *Escherichia coli* and *Salmonella gallinarum* maintain larger cell sizes during the later phases of the culture cycle.

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A COMPARISON OF THE METABOLIC ACTIVITIES OF AEROBACTER AEROGENES, EBERTHELLA TYPHI AND ESCHERICHIA COLI

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Received for publication July 10, 1936

The rate of oxygen consumption per cell in actively growing broth cultures of *Escherichia coli* has been reported by Martin (1932) to reach a maximum value near the end of the lag period of growth at a time when the individual cells have the greatest volume and surface area. Walker and Winslow (1932) reported similar observations on carbon dioxide and ammonia production in peptone and lactose-peptone cultures of *E. coli*, maximum metabolic rates per cell being observed towards the end of the lag period of growth. Walker, Winslow, Huntington and Mooney (1934) concluded that the high rates of carbon dioxide and ammonia production observed in the late lag period of growth could only in part be explained by the increased cell size observed during the same period. Comparison of their results with studies on cell size reported by Henrici (1928), Martin (1932) and Bayne-Jones and Adolph (1932) suggested that while the size of the cells at their maximum near the end of the lag period was not over ten times that at their minimum during the latter phases of growth, yet the rates of carbon dioxide and ammonia production per cell were fifty to one hundred times higher than those observed during the maximum stable period of growth. These observations, together with studies by Mooney and Winslow (1935) on carbon dioxide and ammonia production in cultures of *Salmonella pullorum* and *Salmonella gallinarum* suggested that there is a period of "physiological youth" in bacterial cultures as postulated by Sherman and Albus (1923)

which is characterized not only by larger cells but also by cells that are much more active metabolically per unit of living matter.

Wohlfeil (1930) from his studies on oxygen consumption concluded that the rate of bacterial respiration is higher during the early phases of growth because there are fewer cells present per unit volume and therefore more oxygen is available per organism. An hypothesis similar to that advanced by Wohlfeil was presented by Clifton, Cleary and Beard (1934) in their studies on oxidation-reduction potentials and ferricyanide reducing activities in peptone cultures and suspensions of *E. coli*. Clifton (1936), in a preliminary report, presented additional evidence that the concentrations of the reactants must be considered in any interpretation of the rates of metabolic activity in bacterial cultures.

Since the concentrations of reactants apparently influence the rate of metabolic activity in bacterial cultures as suggested by the studies on oxidation-reduction potentials and ferricyanide-reducing activities in cultures and suspensions of *E. coli*, the studies were extended to include oxygen consumption and carbon dioxide production with the same organism and in the same medium. The studies to be reported here also represent a comparison of these metabolic activities of *E. coli*, and factors influencing their values, with those observed in cultures and in suspensions of *Aerobacter aerogenes* and *Eberthella typhi*.

EXPERIMENTAL

Time-potential relationships

The oxidation-reduction potentials, viable populations and hydrogen ion concentrations developed in aerobic cultures of *Aerobacter aerogenes* (A-5), *Eberthella typhi* (I-4) and *Escherichia coli* (K-12) in a 1.0 per cent Difco peptone, 0.5 per cent sodium chloride medium (pH 7.0) at 37.5°C. were determined by the methods described by Clifton, Cleary and Beard (1934). Typical time-potential, time-population and time-pH relationships observed in stationary and in continuous flow (250 ml. daily) cultures of these bacteria are presented in figure 1.

Ferricyanide reduction and oxygen consumption by samples of stationary and continuous flow cultures

Nine and one-half milliliter samples of the cultures described above were placed in the reduction tubes described by Clifton, Cleary and Beard (1934) and deaerated with nitrogen for one-half hour before the addition of 0.5 ml. of a freshly prepared solution consisting of three parts of 0.1 molar potassium ferricyanide and one part of 0.1 molar potassium ferrocyanide. The rate of

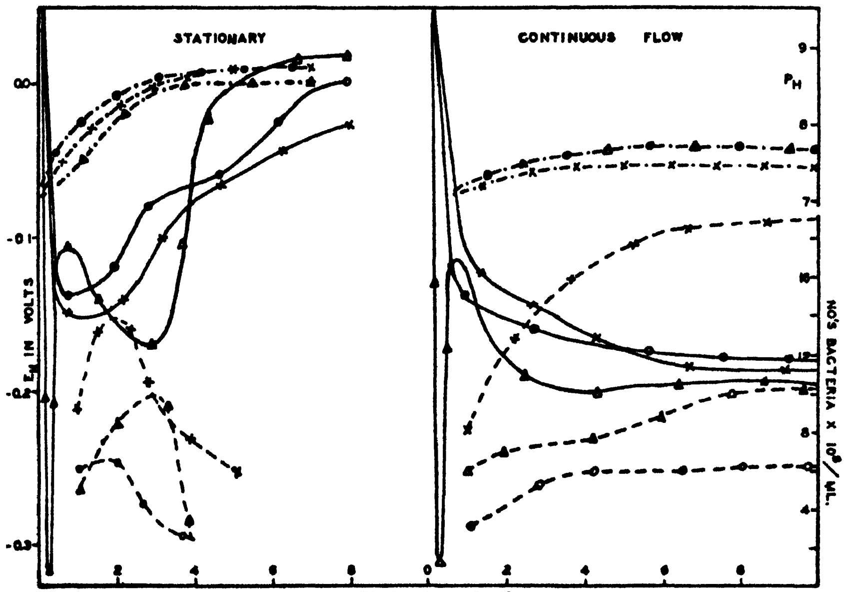

FIG. 1. Time-potential (————), time-population (- - - - - -), and time-pH (.-.-.-) relationships observed in aerobic peptone cultures of *A. aerogenes* (△), *E. typhi* (○) and *E. coli* (×) at 37.5°C.

reduction of the ferricyanide was calculated from the time required to reduce the concentration of ferricyanide from 0.003 to 0.002 M, the ratio of ferricyanide to ferrocyanide and consequently the amount of ferricyanide present at any time being calculated directly from the observed potentials.

Two milliliter samples of the same cultures were placed in Warburg flasks and the oxygen consumption was determined by the usual Warburg technic (1931), the carbon dioxide pro-

duced being absorbed by 5.0 per cent potassium hydroxide. These flasks were shaken for 5 minutes at 37.5° at a rate of 110 cycles per minute with an amplitude of 4 cm. The stopcocks on the manometers were then closed and the vessels shaken for an additional 10 minutes before the first readings were taken. The oxygen consumption was then determined at intervals of 20 minutes. A marked tendency was noted for the oxygen consump-

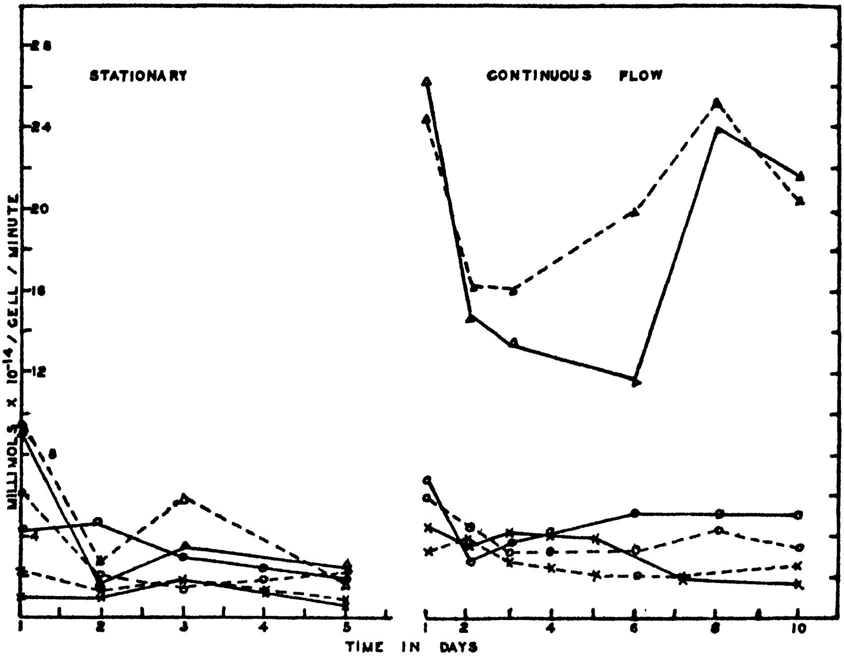

FIG. 2. Millimols $\times 10^{-14}$ of oxygen consumed (————) and of ferricyanide reduced (-----------) per cell per minute by samples from stationary and continuous flow cultures of *A. aerogenes* (△), *E. typhi* (○) and *E. coli* (×) in a 1.0 per cent peptone, 0.5 per cent sodium chloride medium at 37.5°C.

tion of the samples to decrease with time, this decrease being most pronounced in the samples from the older cultures. A marked increase in oxygen consumption was observed when concentrated peptone was added to the cultures, this increase in oxygen consumption of the cultures being generally accompanied by growth of the organisms.

Typical results comparing the influence of the age of the

cultures on the rate of oxygen consumption per cell per minute for the first 20-minute period and the rate of ferricyanide reduction are recorded in figure 2. The oxygen consumption or ferricyanide reduction by filtrates of the cultures was negligible during the test periods.

The results presented in figure 2 indicate that the amounts of oxygen consumed or of ferricyanide reduced per cell by a given culture are of the same order of magnitude. Both the rate of oxygen consumption and of ferricyanide reduction tend to decrease as the average age of the cells increases in the stationary cultures. The oxygen consumption or ferricyanide reduction by stationary cultures older than five days was negligible. As the age of the stationary cultures increased the observed oxidation-reduction potentials also shifted to more positive values than those observed during the period of marked metabolic activity. The potentials in the continuous flow cultures were maintained at a higher reducing intensity and the rates of oxygen consumption and of ferricyanide reduction per cell were in general higher than in the stationary cultures.

Metabolic activity of suspensions of "resting" bacteria

Forty-eight hour cultures of *A. aerogenes*, *E. typhi* and *E. coli*, grown at 37.5°C. on nutrient agar in Kolle flasks, were suspended in physiological saline and washed three times by centrifugation. The cultures were then suspended in mixtures of equal parts of M/15 phosphate buffer, pH 7.4, and physiological saline to give suspensions of approximately the same turbidity. These suspensions were then incubated for 2 hours at 37.5°C. in shallow sterile dishes in order that the cells might utilize any residual foodstuffs.

Measured volumes of these suspensions were introduced into the reduction tubes and appropriate amounts of buffer, saline and substrate added. The concentrations of phosphate buffer and of saline were maintained constant. These suspensions were deaerated with nitrogen for one-half hour before the addition of the 0.075 molar potassium ferricyanide, and 0.025 molar potassium ferrocyanide solution employed in these tests. The results

obtained with typical suspensions of *A. aerogenes*, *E. typhi* and *E. coli*, illustrating the influence of different concentrations of peptone, ferricyanide and viable cells on the rate of ferricyanide reduction are recorded in table 1. The rate of reduction of the ferricyanide was calculated from the time required to reduce the concentration of ferricyanide from 0.003 to 0.002 M.

TABLE 1

Influence of the concentration of peptone, oxidant and organisms on the rate of reduction of $K_3Fe(CN)_6$ at 37.5°C. by suspensions of "resting" bacteria, pH 7.4

Millimols $\times 10^{-14}$ $K_3Fe(CN)_6$ reduced per cell per minute

VIABLE COUNT	A. AEROGENES (13 × 10⁹)	E. TYPHI (13 × 10⁹)	E. COLI (16 × 10⁹)
	Influence of peptone concentration†		
4 0 per cent	11 1	5 8	5 2
2 0 per cent	11 5	5 3	5 2
1 0 per cent	11 5	4 8	5 1
0 5 per cent	8 7	4 3	4.1
0 1 per cent	5 1		1 7
	Influence of initial ferricyanide concentration*		
0 225 millimols	11 9	5 6	5 6
0 150 millimols	11 5	4 8	5 1
0 075 millimols	9 5	1.7	2 8
	Influence of bacterial concentration*†		
1 5 × viable count above	8 6	4 3	4 6
1.0 × viable count above	11.5	4 8	5 1
0 5 × viable count above	13 5	5 4	6 3

* Peptone concentration, 1.0 per cent.

† Initial ferricyanide concentration 0.150 millimols per 20 ml.

Measured volumes of a suspension of well-washed bacteria in the phosphate buffer-saline solution were placed in the Warburg flasks and appropriate amounts of phosphate-buffer saline solution and peptone were added. The concentrations of buffer and saline were maintained constant during the studies on the influence of the concentration of bacteria, peptone or oxygen on

the oxygen consumption of the cells. All results were corrected for oxygen consumption by the medium and by the washed cells.

TABLE 2

Influence of the concentration of peptone on oxygen consumption of a suspension of E. coli during five consecutive twenty-minute periods

Cubic millimeters of oxygen consumed per 20 minutes per 2 ml. of suspension

TIME INTERVAL	CONCENTRATION OF PEPTONE					
	2.0	1.0	0.5	0.25	0.12	0.06
minutes	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
0–20	37.2	36.7	34.7	31.4	23.8	12.8
20–40	59.1	60.0	41.7	33.1	10.6	6.5
40–60	94.4	63.5	52.8	20.0	9.0	2.8
60–80	96.5	63.8	38.8	13.1	3.5	1.4
80–100	114.5	77.6	19.3	8.0	3.5	

TABLE 3

Influence of the initial concentration of bacteria in suspensions of E. coli on oxygen consumption during eight consecutive twenty-minute intervals

Peptone concentration 1.0 per cent

Cubic millimeters of oxygen consumed per 20 minutes per 2 ml. of suspension

TIME INTERVAL	RELATIVE INITIAL NUMBER OF CELLS				
	12	8	4	2	1
minutes					
0–20	77.1	54.9	25.3	15.6	8.3
	(99.6)*	(66.4)	(33.2)	(16.6)	
20–40	76.1	80.3	51.6	27.5	15.8
40–60	109.8	84.2	64.3	35.3	26.5
60–80	126.1	120.6	74.2	43.9	40.8
80–100	137.2	136.0	112.7	68.9	57.8
100–120	86.0	128.8	137.2	103.7	98.8
120–140	75.8	76.8	73.5	83.6	143.3
140–160	38.7	53.2	68.0	44.8	76.6

* Represents amount of oxygen consumed per unit number of cells multiplied by the relative number of cells in the suspension.

The influence of peptone concentration on the amount of oxygen consumed is well illustrated in table 2 in which the

amounts of oxygen consumed in 5 consecutive twenty-minute periods are recorded, the initial numbers of "resting" bacteria being the same in each dilution of peptone.

The influence of the initial concentration of bacteria on the amount of oxygen consumed is illustrated in table 3, the initial concentration of peptone in each flask being 1.0 per cent.

Increasing the concentration of oxygen increased to a slight extent the amount of oxygen consumed per unit time. The amount of oxygen consumed was practically independent of the pH over the range studied, 6.4 to 8.4. The results presented in tables 1 to 3, together with similar results on oxygen consumption obtained with suspensions of *A. aerogenes* and *E. typhi*, illustrate the marked influence of the concentration of peptone and of bacteria on ferricyanide reduction or oxygen consumption by suspensions of these bacteria. The results on oxygen consumption are complicated by the fact that marked growth of the cells occurred in concentrations of peptone greater than 0.25 per cent.

The marked influence of concentrations of peptone less than 0.5 per cent on both the rate of ferricyanide reduction and the rate of oxygen consumption and the influence of peptone concentration on the total oxygen consumption in a 100-minute period by suspensions of these bacteria suggest that the decrease in the rate of metabolic activity of bacteria observed as the age of the culture increases may in part be due to depletion of readily available foodstuffs. The rate of oxygen consumption by suspensions of *A. aerogenes*, *E. typhi* and *E. coli* in filtrates of their respective cultures (adjusted to pH 7.0) was considerably less than in fresh 1.0 per cent peptone and decreased during successive time intervals in a manner analogous to that reported in table 3 for suspensions of *E. coli* in the lower concentrations of peptone. Little or no multiplication of these organisms occurred after inoculation of filtrates of 48-hour or older peptone cultures under the conditions prevailing in the Warburg vessels (low CO_2 tension, marked tendency for pH to shift to more alkaline values) although growth occurred under ordinary test tube conditions.

Marked growth and oxygen consumption was observed in the same filtrates following the addition of small amounts of a concentrated peptone solution. These observations suggested that a more detailed study of the influence of the concentration of peptone on the growth and metabolic activities of these bacteria might aid in explaining the different rates of metabolic activity observed at different phases of the growth cycle.

GROWTH AND OXYGEN CONSUMPTION OF THE ORGANISMS AS INFLUENCED BY DIFFERENT CONCENTRATIONS OF THE REACTANTS

The oxygen consumption of cultures of *A. aerogenes*, *E. typhi* and *E. coli* in different concentrations of peptone was determined by the ordinary Warburg technic. The peptone solutions were inoculated with the test organism and 2-ml. samples were immediately placed in the Warburg vessels which were then attached to the manometers and placed in the water bath at 37.5°C. The gas mixtures in the flasks were replaced with CO_2-free air at frequent intervals during these tests in order to maintain a fairly uniform concentration of oxygen. The flasks were shaken at a rate of 130 cycles per minute with an amplitude of 4 cm. Control tests indicated that a rate of shaking greater than 130 cycles per minute did not appreciably increase the rate of oxygen consumption. Four-tenths of a milliliter of a 20 per cent potassium hydroxide solution was employed in the central cups of the vessels to absorb the carbon dioxide, the absorption of this gas being further facilitated by placing a roll of No. 40 Whatman filter paper in the hydroxide solution. The hydroxide solution was renewed at frequent intervals during the tests. Plate counts were made from duplicate control cultures at one-hour intervals during the period of rapid growth and at longer intervals as the age of the cultures increased. The pH of the cultures rapidly shifted to more alkaline values (pH 8.4 or higher) during the course of these experiments.

The oxygen consumption per cell per minute during the period of rapid growth was computed by the formula of Buchanan (1930) which may be represented as follows:

$$m = \frac{2.303\ S\ (\log b - \log B)}{t\ (b - B)}$$

where m = oxygen consumed per cell per minute;
t = duration of time interval in minutes;
S = oxygen consumed by culture during time t;
B = number of bacteria present at start of time t, and
b = number of bacteria present at end of time t.

Since this equation is valid only during the logarithmic growth phase the arithmetical average of the number of cells present during a given interval of time was employed in calculating the rate of oxygen consumption per cell during the later phases of growth. All observed values for oxygen consumption were corrected for thermo-barometric changes and for oxygen consumption by the sterile media during the test periods.

Typical time-growth, time-oxygen consumption and time-oxygen consumption per cell per minute relationships are recorded in figure 3 for 1.0 per cent Difco peptone, 0.5 per cent sodium chloride cultures of *A. aerogenes*, *E. typhi* and *E. coli*. Similar time-oxygen consumption relationships were observed when the same organisms were grown on a nutrient agar surface in the Warburg flasks.

The oxygen consumption in cultures older than 20 hours (under the conditions of these tests) was of a very low order of magnitude but marked increases were observed following the addition of 0.1 ml. of a 10 or 20 per cent peptone solution to the cultures. This suggests that depletion of readily available foodstuffs may be a limiting factor for growth under the conditions of these tests along with the marked shift in hydrogen ion concentration of the cultures to pH values of 8.5 or higher.

The studies were extended to include the influence of a wide range of peptone concentrations (0.1 to 10.0 per cent), of filtrates of these cultures and of oxygen on the oxygen consumption of these organisms. Typical results obtained with *E. coli* as the test organism are presented in figure 4, together with a summary of the results obtained with *A. aerogenes* and *E. typhi*. All tests reported in figure 4 were conducted in the presence of air with

the exception of one culture in 5.0 per cent peptone in an atmosphere of oxygen.

The rate of growth of the bacteria increased slightly as the concentration of peptone or of oxygen was increased (same initial numbers of bacteria) while the total crop in 24 hours increased approximately four times as the concentration of

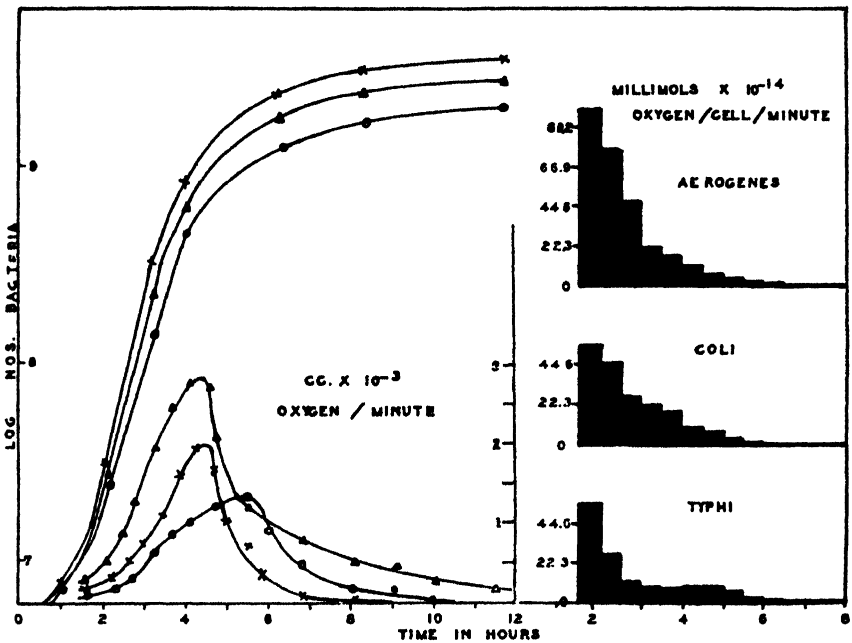

FIG. 3. Time-growth, time-oxygen consumption per minute per milliliter of culture and time-oxygen consumption per cell per minute relationships observed during the growth of *A. aerogenes* (△), *E. typhi* (○), and *E. coli* (×) in a 1.0 per cent peptone solution at 37.5°C. (44.6 × 10^{-14} millimols oxygen = 1.0 × 10^{-11} cc.).

peptone was increased from 1.0 to 10.0 per cent. The substitution of oxygen for air in the flasks markedly increased the oxygen consumption in the early phases of growth. However, the oxygen consumption fell to a low level earlier in the growth curve in the presence of oxygen as compared with air.

The total amounts of oxygen consumed by the three test organisms during the first ten hours in 1.0, 5.0 and 10.0 per cent

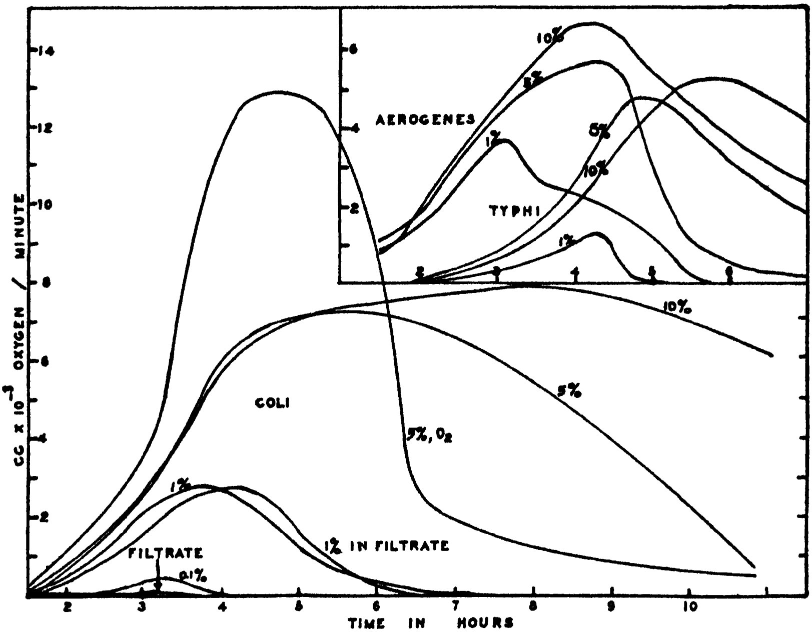

FIG. 4. The influence of peptone concentration, of a 48-hour filtrate (adjusted to pH 7.0) of a 1.0 per cent peptone culture of *E. coli* and of oxygen on the oxygen consumption per minute per milliliter by cultures of *E. coli*. The influence of peptone concentration on the oxygen consumption of *A. aerogenes* and of *E. typhi*.

TABLE 4

The influence of peptone concentration on the amounts of oxygen consumed per milliliter of cultures of A. aerogenes, E. typhi and E. coli during the first ten hours of growth in Warburg vessels at 37.5°C.

Cubic millimeters of oxygen consumed per milliliter of culture

ORGANISM	CONCENTRATION OF PEPTONE IN PER CENT		
	1.0	5.0	10.0
A. aerogenes	510	1,300	1,500
E. typhi	180	800	1,400
E. coli	430	2,400	3,300

peptone cultures were estimated from the graphs. The amounts of oxygen consumed per milliliter of the various cultures during the first ten hours are summarized in table 4.

In addition to the studies on oxygen consumption, the carbon

dioxide production of cultures of *E. coli* at 37.5°C. in 1.0, 5.0 and 10.0 per cent peptone was determined by the methods described by Walker (1932). Typical results are presented in figure 5.

The studies on the rate of carbon dioxide production by cultures of *E. coli* in peptone solutions of different concentrations show the same general time-activity relationships as observed in the studies on oxygen consumption and again illustrate the

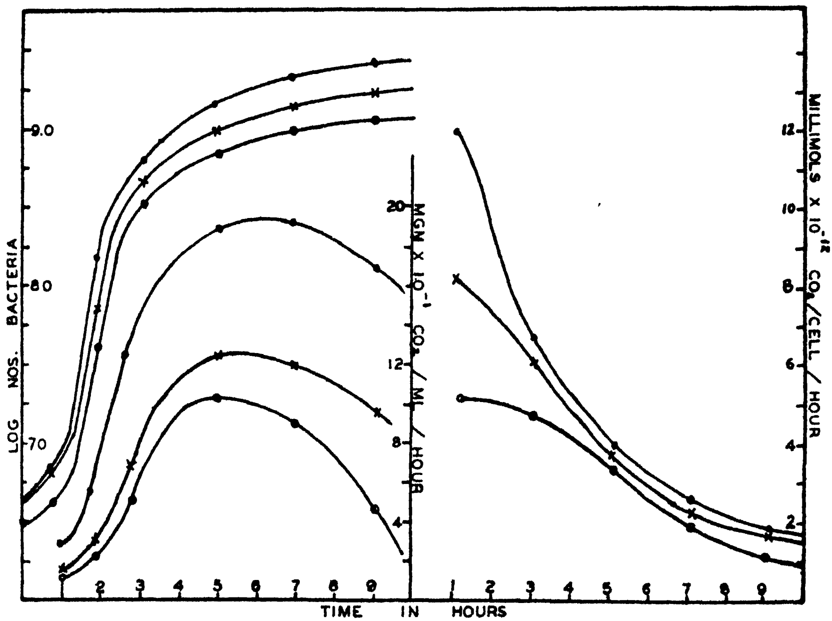

FIG. 5. GROWTH AND CARBON DIOXIDE PRODUCTION IN AERATED PEPTONE CULTURES OF *E. coli* AT 37.5°C.

1 per cent peptone (O——O), 5 per cent peptone (X——X) and 10 per cent peptone (●——●)

marked influence of concentration of foodstuffs on the metabolic activities of bacteria. The carbon dioxide production in 24-hour, 1.0 per cent peptone cultures of *E. coli* was markedly higher than the oxygen consumption in similar cultures as determined by the Warburg technic. However, the pH was only 7.5 in the 24-hour aerated culture employed in the carbon dioxide production tests as compared with 8.5 or higher in the oxygen consumption tests.

DISCUSSION

The time-potential relationships observed in ordinary and continuous flow cultures of *E. typhi* and *A. aerogenes* are similar to those previously reported for *E. coli*, with the exception of the high reducing conditions ($-0.320\ v$) temporarily developed during the early stages of growth in cultures of *A. aerogenes*. These cultures also show the same general tendency to develop lower reducing intensities as the numbers of viable bacteria decrease in the stationary cultures and to maintain the reducing intensity quite constant and at a relatively high level in the continuous flow cultures.

The general tendency for the rate of reduction of ferricyanide per cell to decrease with increasing age of the cells, as previously reported for *E. coli*, is also observed in cultures of *A. aerogenes* and *E. typhi*. The highest rate of reduction in the cultures tested is observed in cultures of *A. aerogenes*. The rate of reduction per cell in cultures of *E. typhi* is higher than in similar cultures of *E. coli* but the studies with "resting" bacteria indicate that the order would be reversed if the actual concentrations of viable cells were considered.

The rate of reduction of ferricyanide per cell increases as the concentration of peptone or of ferricyanide is increased. This increase is not proportional to the concentration of peptone or of ferricyanide and holds only within certain limits, due to an apparent inhibitory action of high concentrations of peptone or of ferricyanide. The rate of reduction of ferricyanide per cell decreases as the concentration of bacteria is increased, within the range studied, other factors being maintained constant.

The rates of oxygen consumption per cell in samples of the stationary cultures are approximately equal to the rates of ferricyanide reduction and both tend to decrease with increasing age of the cells. The rates of oxygen consumption per cell are in general higher in the continuous flow cultures than in the stationary cultures and tend to remain at a fairly constant level after maximum growth is established.

The rate of oxygen consumption per cell in suspensions of the test organisms increases as the concentration or peptone is

increased, this increase being most marked when the concentration of peptone is less than 0.25 per cent, while the rate of oxygen consumption per cell tends to decrease as the concentration of bacteria is increased. These results, while somewhat complicated by growth of the cells during the tests, lend further support to the theory that the rate of the metabolic activities per cell in a given culture under favorable conditions is controlled by the concentrations and nature of the foodstuffs, oxidants and bacteria and that these factors play closely connected rôles in regulating growth and oxidation-reduction potentials in bacterial cultures.

The results of these studies on oxygen consumption and carbon dioxide production by cultures of *A. aerogenes*, *E. typhi* and *E. coli* are in general agreement with those reported by Martin (1932) and by Winslow and his coworkers (1932–1935) for cultures of *E. coli*. The rate of oxygen consumption or of carbon dioxide production per cell increases rapidly from the time of inoculation to a point of maximum activity near the end of the lag phase or early in the logarithmic period of the growth curve. A limited number of observations indicate that these bacteria also have a maximum size near this time. A marked decrease in the metabolic rates per cell is observed as the age of the cultures increases. However, the maximum rate of oxygen consumption or of carbon dioxide production per unit volume of the cultures is observed early in the negative acceleration in growth phase of the growth curve in 1.0 per cent peptone and later in the growth curve when the concentration of peptone is increased to 5.0 or 10.0 per cent.

Attempts to explain the observed differences in the rate of metabolic activity at various phases of the growth cycle on the basis of changes in cell size or in physiological activity of the cells have not been completely satisfactory. The first factor undoubtedly plays an important rôle in controlling the rates of metabolic activity per cell but the concentrations of the reactants must also be considered in any interpretation of studies on the rates of metabolic activity in bacterial cultures. As the numbers of bacteria increase in a culture, the total amount of oxygen consumed, of carbon dioxide produced or of ferricyanide

reduced per unit time increases to a maximum value determined by the concentrations of the reactants, the nature and size of the organisms and the physical and chemical influence of the environment on the cells. The decrease, increasing with age of the culture, in the rate of metabolic activity per cell may be interpreted on a probability basis. As the number of bacteria increases the concentration gradient of foodstuffs and of oxidants between the cell and its environment decreases. Therefore, the probability of sufficient materials being available per cell per unit time to provide, upon interreaction, for the maximum requirements of the cells decreases. The addition of concentrated foodstuffs to a culture in the later phases of growth increases the foodstuff concentration gradient per cell and produces an increase in the metabolic activities of the cells. This increase, however, is only of limited duration due to the high total energy demand of the large number of cells in the cultures.

The results obtained in the studies on oxygen consumption indicate the marked influence of the concentration of peptone, of bacteria and of oxygen on the metabolic activities of the cells and suggest that, within limits, an increase in concentration of foodstuff or of oxidant, increases the probability of sufficient materials being available per cell per unit time to meet the maximum requirements of the cell. The rapid decrease in the observed oxidation-reduction potentials in the cultures during the early phases of growth together with the maximum reducing intensity developed in or near the maximum stationary growth phase also suggest the marked influence of concentrations of reactants on the metabolic activities of the cells. Longsworth and MacInnes (1936) have presented evidence that there is a correlation between the observed oxidation-reduction potentials and the rate of metabolic activity (acid production at constant pH) in cultures of *Lactobacillus acidophilus*. The rate of acid production per cell in their cultures decreased markedly during the growth period, the generation time tended to increase and the observed potentials rapidly fell to more negative values. An increase in the initial concentration of sugar markedly increased the rate of acid production.

These various observations suggest that growth may be primarily controlled by the probability relationships discussed above (see also Cleary, Beard and Clifton, 1935). The growth rate decreases as the concentration per cell of materials essential for growth decreases in cultures of bacteria in which a relatively high population has been established. These results also suggest that the maximum population developed under favorable conditions, particularly pH and concentration of waste products, may be primarily controlled by the concentration relationships.

SUMMARY AND CONCLUSIONS

Growth, oxidation-reduction potentials, ferricyanide reduction, oxygen consumption and carbon dioxide production have been studied in cultures and in suspensions of *Aerobacter aerogenes*, *Eberthella typhi* and *Escherichia coli*.

The concentrations of peptone, organisms and oxidant play closely connected rôles in controlling the metabolic activities of the cells, as measured by the metabolic indices mentioned above.

The various observations suggest that the rate of bacterial metabolism per cell is highest during the early phases of growth because of the increased size of the cells and the higher concentration gradient of foodstuffs between the cells and their environment.

Additional support is presented for the hypothesis that the growth rate and the maximum population developed in a favorable environment may be primarily controlled by the probability of sufficient materials being available per cell per unit time to provide for the metabolic requirements of the cell.

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THE FACULTATIVE SPORULATING BACTERIA PRODUCING GAS FROM LACTOSE

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Received for publication July 11, 1936

Facultative spore-bearing bacilli capable of fermenting carbohydrates with the formation of gas in the Durham tube have been reported by many investigators. Such organisms, once thought to be rare, appear to be ubiquitous for they have been isolated from water, soil, human and animal feces, sewage, eggs, decaying and canned vegetables, and grains. The bacteria of this group have attracted attention because of their ability to produce considerable quantities of acetone and ethyl alcohol from carbohydrates, and also on account of their occasional occurrence in water supplies. Since they are Gram-negative and usually do not form spores on carbohydrate media, they have been a source of confusion in the interpretation of bacterial water analysis for the incidence of the colon group.

There is some confusion in the literature concerning the identity and differentiation of members of this group. This report presents briefly a study of all available strains described in the literature, together with a number of freshly isolated cultures.

Donker (1926) proposed that a new genus be created to include the facultative sporulating bacteria that are motile by means of peritrichous flagella, form clostridia, produce catalase and ferment carbohydrates with the production of gas. He suggested the generic name *Aerobacillus* for these organisms which he considered as having some of the characteristics of the genera *Aerobacter* and *Bacillus*. However, Pribram (1929 and 1933) employed the generic name *Aerobacillus* to include only the aerobic bacteria which are motile by means of peritrichous flagella, bear terminal spores, and are Gram-negative.

From the description of the organisms listed in this genus by Pribram, it is evident that they do not conform to the characterization of the genus *Aerobacillus* of Donker. On the other hand, such organisms as *Bacillus asterosporus* and *Bacillus polymyxa*, listed by Donker in the genus *Aerobacillus*, are allocated by Pribram to his genus *Bacillus*. It is apparent, therefore, that the generic designation *Aerobacillus* as employed by Donker and by Pribram does not refer to the same group of organisms. Due to priority of Donker, his designation should be adopted if the genus *Aerobacillus* is found to be desirable. It is in this latter sense that the term will be employed in this contribution. (Bergey (1934) includes these species in the genus *Bacillus*.)

Five species of the genus were described by Donker, namely:

1. *Aerobacillus polymyxa* (Prazmowski) Donker
   Syn: *Clostridium polymyxa* Prazmowski
   Granulobacter polymyxa Beijerinck
   Bacillus polymyxa Beijerinck and den Dooren de Jong
   Bacillus asterosporus (A. Meyer) Migula
2. *Aerobacillus acetoethylicus* (Northrop) Donker
   Syn: *Bacillus acetoethylicum* Northrop
3. *Aerobacillus macerans* (Schardinger) Donker
   Syn: *Bacillus macerans* Schardinger
4. *Aerobacillus violaris* (Bréaudat) Donker
   Syn: *Bacillus violarius acetonicus* Bréaudat
5. *Aerobacillus amaracrylus* (Voisenet) Donker
   Syn: *Bacillus amaracrylus* Voisenet

A brief review of the literature concerning each organism listed above, is presented to facilitate a proper understanding of this group of bacteria.

1. AEROBACILLUS POLYMYXA

Prazmowski (1880) described an organism that closely resembled *Clostridium butyricum* but could grow in the presence of air. Starch and cellulose were strongly attacked, and carbon dioxide gas was formed from some carbohydrates. The organism was designated as *Clostridium polymyxa*. Beijerinck (1893 and 1896) studied an organism that grew best aerobically and

produced slime in carbohydrate media. Gases were formed, and upon analysis were found to consist of carbon dioxide and small amounts of hydrogen. The organism was described as rod-shaped and motile, and produced spores and granulose. Its discoverer placed it in the genus *Granulobacter* (*Granulobacter polymyxa*) and reported it to be found "normally on grains of wheat and very plentiful in garden soil." In later studies of this organism, Beijerinck and van Delden (1902) reported cultural differences which led them to recognize two varieties, namely, *Granulobacter polymyxa* var. *mucosum* and *Granulobacter polymyxa* var. *tenax*.

Gruber (1905) isolated an organism from milk which he thought was identical with *Clostridium polymyxa*. Growth was best in the absence of air but spores were formed only under aerobic conditions.

Meyer (1892) isolated from carrots an organism to which he gave the name *Astasia asterospora* because of its ridged spore resembling a star. The organism was unusual also in that it presented a new type of arrangement of flagella, and that a nucleus was observed in the cells. These peculiarities attracted the attention of Migula (1898) who obtained a culture for study, but was unable to confirm the observations of Meyer. Subsequently, Meyer (1899) acknowledged his previous error and confirmed the work of Migula. Aderhold (1899) observed *Astasia asterospora* in canned asparagus.

Migula (1900) included *Astasia asterospora* in the genus *Bacillus*, listing it as *Bacillus asterosporus* (Meyer) Migula. In 1901, Gottheil suggested that probably *Bacillus subanaerobius* Gruber (1887) and *Bacillus thalassophilus* Russell (1892) were synonymous with *B. asterosporus* (Meyer) Migula.

Behrens (1902, 1903) observed that *B. asterosporus* in pure culture would ret flax and hemp. Chester (1903) studied a culture of *B. asterosporus*, supposedly isolated by Meyer, and gave a fairly complete description of its characteristics. In 1903 Meyer again published results of his researches on this organism. It is interesting that he reported, contrary to other workers, that most of the gas produced by this culture was hydrogen

rather than carbon dioxide. Wund (1906) observed that an atmosphere containing 100 mgm. of oxygen per liter was optimum for spore formation of *B. asterosporus*. This work was later confirmed by Meyer (1909).

Bleau (1905) reported the optimum temperature for *B. asterosporus* to be about 35°C., with spore formation largely inhibited at 40° to 45°C.

B. asterosporus was found in the intestinal canal of cattle by Ankersmit (1906) and in decaying vegetables by Wahl (1906). Hasselhoff and Bredemann (1906) isolated a number of organisms from vegetables and named three new species, *Bacillus clostridioides*, *Bacillus dilaboides* and *Bacillus asterosporus* (*alpha*). In a later publication Bredemann (1909b) concluded that these forms were sufficiently alike to be considered as one species, *B. asterosporus*.

Other workers who have reported researches concerning *B. asterosporus*, but whose contributions space does not allow us to consider here, are Garbowski (1907), Ritter (1908), Bredemann (1909c), Meyer (1909), Eisenberg (1909), Viehover (1912), Barthel (1922), Lisk (1923), Virtanen and Karström (1925), McFall (1929), Stapp and Zycha (1931), Patrick (1931) and Zycha (1932).

2. AEROBACILLUS ACETOETHYLICUS

In an attempt to find a cheap method for the production of acetone, Northrop, Ashe and Senior (1919) isolated from decaying potatoes an organism which they named *Bacillus acetoethylicum*, since its most striking characteristic was the formation of acetone and ethyl alcohol. The organism was described in detail as a motile sporulating rod, and Gram-negative, a facultative anaerobe, with extensive fermentative powers. Solutions of molasses served as a satisfactory substrate for the organism, and fairly large quantities of acetone and ethyl alcohol were obtained when the reaction was adjusted to pH 8.5 to 9.5 (Northrop, Ashe and Morgan (1919)).

Peterson and Fred (1920), studying intermediate products of the fermentation of carbohydrates, noted that acetaldehyde was

produced by the acetone-forming organism, *B. acetoethylicum.* The products of fermentation of various substrates by *B. acetoethylicum* have been investigated by Arzberger, Peterson and Fred (1920); Peterson, Fred and Verhulst (1921); Juritz (1921); Fred, Peterson and Anderson (1923); Speakman (1925); Bakonyi (1926); and Patwardhan (1930).

Donker (1926) recognized *Aerobacillus acetoethylicus* as a separate species, although his description showed that it was closely related to *Aerobacillus polymyxa* and *Aerobacillus macerans.*

3. AEROBACILLUS MACERANS

In 1904 Schardinger described a rod-shaped spore-bearing facultative organism that produced gas and quantities of acetone and ethyl alcohol from carbohydrates. The following year (1905) he published a more detailed description of the organism and gave it the name *Bacillus macerans* (bacillus of retting). Other researches in which he studied the fermentative behavior of this culture were published by Schardinger in 1907, 1909 and 1911. Euler and Svanberg (1922), in a study of the effect of reaction on the growth of *B. macerans* and the course of starch splitting, found the optimum acidity for the growth of the organism in the starch medium to be about pH 6.8.

Hinman and Levine (1922) isolated a number of strains of facultative, spore-forming, lactose fermenting organisms from Iowa surface waters. The morphological and biochemical characteristics of the strains caused the writers to conclude that they were dealing with *B. macerans* or *B. acetoethylicus.*

Coles (1926), in a study of the digestion of pectin and methylated glucoses by various organisms, employed two strains isolated by Hinman and Levine and reported that both fermented pectin with the production of acid and gas. The decomposition of starch by *B. macerans* was studied by Samec (1927). Burkey (1928), in a study of the fermentation of cornstalks and their constituents, isolated two organisms which differed from those isolated by Hinman and Levine only in that they liquefied gelatin.

Zacharov (1930) reported that *B. macerans* produces only

ethyl alcohol, never butyl alcohol, and that the production of acetone and ethyl alcohol is in ratio of one to two.

Meyer (1935) observed that organisms of the *B. macerans* group were present in his crude cultures of cellulose-decomposing organisms.

Donker (1926) listed *B. macerans* as one of the five species of the genus *Aerobacillus*.

4. AEROBACILLUS VIOLARIS

Bréaudat (1906) isolated from polluted water an organism which he described as rod-shaped, Gram-negative (at three days), motile, facultative, spore-bearing, and producing acetone from sucrose. A deep violet pigment was formed on potato and on agar media in the presence of peptone and air. He named the organism *Bacillus violarius-acetonicus*.

The description by Bréaudat is incomplete; and there is a possibility that his organism does not belong with the other species discussed in this paper. A very important characteristic possessed by all species of the genus *Aerobacillus* is the ability to produce gas in the decomposition of carbohydrates. Bréaudat failed to mention whether his organism possessed this characteristic, and the culture is no longer available. Dr. A. R. Prévot, Chief of the Laboratory at the Institute Pasteur, stated in a private communication that this organism was no longer alive.

5. AEROBACILLUS AMARACRYLUS

Voisenet (1911, 1913, 1914, 1918) described an organism which he found in water and also in bitter wines. He studied particularly its ability to dehydrate glycerol with the formation of acrolein, and because of this interesting characteristic he called it *Bacillus amaracrylus* (1913). The organism was described as rod-shaped, spore-bearing, motile and Gram-positive, and produced gas in the fermentation of carbohydrates.

Warcollier and LeMoal (1932) and Warcollier, LeMoal and Tavernier (1934) noted the presence of acrolein in cider and wine and concluded that the responsible organism was similar to *B. amaracrylus* or to *Clostridium welchii*.

Donker (1926) and McFall (1929) included Voisenet's species in the genus *Aerobacillus*. No experimental work was done with the organism by these authors and we have been unable to obtain a culture for the present study.

6. OTHER "AEROBACILLI"

A number of organisms have been described in the literature which seem to possess the essential characteristics of the genus *Aerobacillus* Donker. Only a brief review of these reports is necessary.

Wagner (1916) isolated an organism from eggs that he named *Bacillus mycoides* var. *ovoaethylicus*, and which Perlberger (1924) described as a motile rod, spore-bearing, and producing acid and gas in a number of carbohydrates, polyatomic alcohols and glucosides. Perlberger concluded that the organism was not one of the *Bacillus mycoides* group, but was closely related to *B. asterosporus*. Pribram (1933) stated that the Wagner culture was similar to *B. polymyxa* and designated it *B. ovoaethylicus*.

Greer and his co-workers (1928) in a series of papers dealing with the sanitary significance of lactose-fermenting bacteria not belonging to the *Bacillus coli* group, described organisms that were aerobic, spore-forming and fermented lactose with the production of acid and gas. The name *Bacillus aerosporus* was given to the group. In their work about sixty strains were used, all of which were very similar and differed little from previously described organisms. They were able to isolate *B. aerosporus* from 17 out of 18 samples of horse manure, 11 out of 14 samples of cow dung, 1 out of 18 samples of human feces, 3 out of 44 samples of sewage, and 7 out of 9 samples of fertilized soil.

Coolhaas (1928) isolated and described an organism very similar to that of Schardinger's, except that it was more thermophilic. He called it *Bacillus thermoamylolyticus*.

Bacillus pandora was isolated from *Hevea latex* by Corbet (1929, 1930) and described as spore-forming, facultative, and producing gas in the fermentation of lactose and sucrose.

Bacterium hessii was isolated from slimy milk by Guillebeau (1891) and was reported to produce some gas in milk agar. The

organism was transferred to the genus *Bacillus* by Flügge (1896), and Neide (1904) stated that it was probably synonymous with *Bacillus silvaticus*. The meager description of the original culture does not justify placing it in the group of "aerobacilli," and the organism is no longer available for study.

Meyer (1918), Ewing (1919), Ellms (1920), Weight (1924), Norton and Weight (1924), Ginter (1927), and Koser and Shinn (1927), isolated and described organisms which, because of their ample descriptions, can undoubtedly be considered as belonging to the "aerobacilli." Other writers who have probably noted the occurrence in their studies of aerobic spore-forming bacteria fermenting carbohydrates with gas production, are: Burton and Rettger (1917), Hall and Ellefson (1918 and 1919), Perry and Monfort (1921), Havens and Dehler (1923), Raab (1923), Sohn (1924) Gettrust and Hostetter (1925 and 1930), Berry (1925), and Janzig and Montank (1928).

Zeissler (1930), Hall (1935) and others have observed that certain anaerobic species of bacilli are capable of delicate aerobic growth on agar media. Some of these microaerophilic organisms, such as *Bacillus carnis* and *Clostridium tertium*, resemble the "aerobacilli" in that they ferment carbohydrates with gas production. The species mentioned differ from those in the "aerobacillus" group in that catalase is not produced, and growth is almost completely inhibited by free oxygen, except on media reduced by the addition of blood or some other reducing material. Hall reported that sporulation of the "microaerophilic" anaerobes was inhibited by free oxygen. The aerobacilli, on the other hand, sporulate readily under aerobic conditions. Therefore, it seems evident that the oxygen-tolerant anaerobic species referred to, do not belong in the genus *Aerobacillus* Donker.

SOURCE OF CULTURES

In this study all available cultures having the characteristics of the genus *Aerobacillus* Donker were employed. Some of the acquired cultures failed to conform to previous descriptions which would place them in the group of "aerobacilli." *B. macerans* (Berlin) and *B. asterosporus* 62a (Apia, Samoa), secured from the

Pribram collection, failed to produce gas from any carbohydrates and were therefore not included. Also, the culture of *B. thermoamylolyticus* received from N. L. Söhngen, did not satisfy the requirements of the genus and was not used in this study. Table 1 shows the source, designation and species allocation of the cultures studied. All named species of the genus were available for study except *B. violarius-acetonicus* Bréaudat and *B. amaracrylus* Voisenet, which are apparently no longer in existence.

In addition to the previously described organisms, 63 strains isolated in this laboratory were studied. These strains were obtained from various sources and purified by the serial dilution pour plate method. Because of the slimy nature of most of the organisms, each strain was replated at least twenty times.

MORPHOLOGY

Smears prepared from glucose agar and nutrient agar cultures after incubation for 18 hours, 36 hours, 3 days and 7 days, were stained with methylene blue and by Gram's method (Hucker modification). Cell measurements were made with a Filar micrometer. All cultures studied were rod-shaped and varied from approximately 2.5 to 6.0 microns in length and from 0.6 to 1.1 microns in width. On media containing fermentable sugar the cells were somewhat larger than on sugar-free media. The endospores were elliptical, about 0.8 by 1.4 microns, and when seen in the cells were located terminally or subterminally. Spores were not observed in glucose agar cultures. All strains were motile when hanging drop examinations were made of 16-to-18 hour (37°C.) nutrient broth cultures. All cultures were Gram-negative at 18 hours (37°C.).

CULTURAL CHARACTERISTICS

Plain nutrient agar. Moderate, spreading, effuse transparent growth; medium unchanged; no distinct odor and no chromogenesis.

Glucose agar. Some strains produced growth comparable to that on plain nutrient agar, while other strains grew abundantly and produced raised slimy colonies.

TABLE 1

Source, designation and species allocation of faculative, sporulating, aerogenic bacilli

SOURCE	NUMBER OF STRAINS	ORIGINAL NAME	ISOLATED BY	RECEIVED FROM	SPECIES ALLOCATION
Psyllium seed	1		McCleskey (1931)		*Aerobacillus macerans*
Grains	5		Porter (1933)		
Sewage	1		Porter (1933)		
Lake water	1		Porter (1933)		
Potato	2	*B. acetoethylicum*	Northrop, et al. (1917)	Amer. T. C. C.	
Potato	1	*B. acetoethylicum*	Northrop, et al. (1917)	J. H. Northrop	
Potato	1	*B. macerans*	Schardinger (1904)*	Amer. T. C. C.	
Potato	1	*B. macerans*	Schardinger (1904)*	Pribram, Vienna	
Water	2	*B. macerans*	Hinman-Levine (1921)	I. S. C. Lab.	
Water	1			O. K. Stark	
Psyllium seed	21		McCleskey (1931)		
Canned rhubarb	1		McCleskey (1934)		
Soil	15		McCleskey, Porter (1933)		
Grains	2		Porter (1933)		
Soil	7	*Aerobacillus polymyxa*	McFall (1928)	I. S. C. Lab.	*Aerobacillus polymyxa*
Corncobs	2	*Aerobacillus polymyxa*	McFall (1928)	I. S. C. Lab.	
Spinach	1	*Aerobacillus polymyxa*	McFall (1928)	I. S. C. Lab.	
Cauliflower	1	*Aerobacillus polymyxa*	McFall (1928)	I. S. C. Lab.	
Beets	1	*Aerobacillus polymyxa*	McFall (1928)	I. S. C. Lab.	
Potato	4	*Aerobacillus polymyxa*	McFall (1928)	I. S. C. Lab.	

Endive, malt, lettuce	4	*Aerobacillus polymyxa*	Donker (1926)*	Amer. T. C. C.	*Aerobacillus polymyxa*
Water	2			Amer. T. C. C.	
Soil	1		Weight (1926)	Univ. Utah	
Human feces	1		Weight (1926)	Univ. Utah	
Water	1		Ginter (1925)	Univ. Utah	
Eggs	1	*B. mycoides* var. *ovoa-ethylicus*	Wagner (1916)*	Pribram, Vienna	
Soil	1	*B. asterosporus*	Bredemann (1909)*	Pribram, Vienna	
Carrots	1	*Astasia asterospora*	A. Meyer (1892)*	Pribram, Vienna	
Water	1	*B. aerosporus*	Greer, et al. (1928)*	Chicago Bd. H. Lab.	
Water	2		Kauffmann (1934)*	J. Smit, Amsterdam	
Soil	1	*B. asterosporus*	Bredemann (1909)*	Univ. Marburg	

* Date of publication.

Broth. Slight clouding and very little sediment. In sugar broth, some of the cultures produced slime.

Gelatin. Scanty to moderate filiform growth along the line of puncture (20°C.). Strains which liquefied gelatin produced crateriform liquefaction.

Potato. Many strains were able, in 48 hours at 37°C., to reduce potato to a soft pulp. Some strains however lacked this strong diastatic action. A distinct fruity odor was produced. The color of the growth on potato varied from white to light tan.

Loeffler's blood serum. Scanty, effuse growth, with no change in the medium.

Colony characteristics. On plain agar, surface colonies were irregular in form, usually smooth, effuse, and transparent, with no distinct internal structure. Subsurface colonies were circular or elliptical, with entire edge, and granular internal structure. On sugar agar with china-blue indicator there was considerable strain variation in type of colony. Some strains produced round convex slimy colonies, while others produced colonies which were flat and amoeboid. All colonies produced acid. The colonies produced on Endo's agar were similar to those described for china-blue agar. On eosine methylene-blue agar, however, growth of all strains was almost prevented; pinhead colonies with distinct metallic sheen were present after 48 hours at 37°C. Growth on blood agar was abundant with only two strains producing slight hemolysis after 24 hours at 37°C. Colony characteristics were not correlated with the physiological differences noted below.

PHYSIOLOGICAL CHARACTERISTICS

Temperature relations. Sucrose broth, in Durham fermentation tubes, and nutrient broth were incubated at 13°, 20°, 30°, 37°, 42°, 45°, and 50°C., until the medium reached a constant temperature. The tubes were then inoculated and replaced in the incubators at the indicated temperatures. The results of the experiment are given in table 2. On the basis of temperature requirement for growth, the cultures fall into two groups. Those which grew well at 42° to 45°C., but poorly if at all at 20°C., will

be designated as the "*macerans*" group; those that grew luxuriantly at 20°C., but slowly if at all at 42° to 45°C., will be referred to as the "*polymyxa*" group.

Oxygen relationship. For anaerobic studies, McIntosh and Fildes jars were employed. The cultures grew on plain nutrient agar slants under either aerobic and anaerobic conditions, hence they are considered as aerobic and facultative. The "*macerans*" group grew somewhat more luxuriantly under anaerobic conditions than did the "*polymyxa*" group.

Acetyl methyl carbinol production was determined in Bacto M.R.-V.P. medium after incubation for 3 days at 37°C. The indicator employed was the O'Meara reagent as modified by Levine, Epstein and Vaughn (1934). The cultures fall into two groups based on the production of acetyl methyl carbinol. The

TABLE 2

Temperature relations of facultative, sporulating, aerogenic bacilli

SPECIES ALLOCATION	PER CENT POSITIVE (SHOWING GROWTH)					
	13°C 1 week	20°C 24 hours	37°C. 24 hours	42°C 24 hours	45°C 24 hours	50°C 24 hours
Aerobacillus polymyxa	100	100	100	0	0	0
Aerobacillus macerans	0	0	100	100	100	0

strains of the "*polymyxa*" (low temperature) group produced acetyl methyl carbinol, whereas the "*macerans*" (high temperature) group did not.

Relationship to reaction of medium. All the strains were inoculated into glucose broth, adjusted and buffered at pH values of 3.4, 4.4, 5.6, 6.0, 7.0, 8.0, and 8.5. All of the organisms grew well within 48 hours in all the media except those at pH 3.4 and 4.4. No growth occurred in the latter media after two weeks incubation at 37°C. The upper limits of pH supporting growth were not determined.

Indol production was determined in Bacto tryptophane broth after incubation at 37°C. for 3, 5 and 7 days. Kovac's reagent was employed as the indicator. All strains were negative.

Production of hydrogen sulphide. To detect hydrogen sulphide

production three media were tried: Bacto Kligler lead acetate agar; the medium of Patrick and Werkman (1933); and the medium proposed by Levine, Vaughn, Epstein and Anderson (1932). None of the cultures produced hydrogen sulphide in the media employed.

Reduction of nitrate to nitrite was determined in a 0.1 per cent peptone solution to which was added 0.02 per cent KNO_3 and 0.05 per cent NaCl. Incubation was at 37°C. for 3 days, and the test reagent employed was sulphanilic acid and naphthylamine-acetate. All the strains reduced nitrate to nitrite. Gas was not produced.

Litmus milk. All of the cultures decolorized the litmus and produced acid and gas. None of the "*macerans*" group coagulated the milk or caused visible peptonization, whereas most of the "*polymyxa*" strains caused both coagulation and partial digestion (table 4).

Utilization of simple triglycerides and natural fats. The method used was that of Collins and Hammer (1934). The substances tried were tri-propionin, butter fat, lard, and cottonseed oil. Incubation was at 20° and 37°C. for three days or longer. None of these materials were utilized.

Utilization of citric and malonic acids. Bacto Koser's citrate medium and Leifson's sodium malonate medium were employed. None of the strains were able to utilize the salts of the organic acids under the conditions of the experiment.

Fermentation reactions. Standard Durham fermentation tubes were employed to determine the ability of the cultures to produce acid and gas in the following substances: *l*-arabinose, *d*-xylose, rhamnose, *d*-glucose, galactose, levulose, *d*-mannose, lactose, maltose, melibiose, sucrose, trehalose, cellobiose, raffinose, melezitose, starch, dextrin, glycogen, inulin, pectin, xylan, aesculin, amygdalin, salicin, saponin, *a*-methyl-glucoside, glycerol, erythritol, adonitol, mannitol, sorbitol and dulcitol.

Of the above compounds, erythritol, adonitol, dulcitol and inositol were not fermented by any cultures. The "*macerans*" (high temperature, V.P. negative) group fermented sorbitol and rhamnose with the production of acid and gas, where the "*poly-*

myxa" (low temperature, V.P. positive) group failed to ferment either. All the other substances were attacked, and acid and gas were produced by all strains of both groups, except three strains of the "*polymyxa*" group which did not ferment glycerol.

SEROLOGY

Pathogenesis. These organisms are considered to be non-pathogenic for rabbits. Two strains of the "*macerans*" group and 9 strains of the "*polymyxa*" group produced no symptoms of disease when living cultures were injected intravenously into rabbits. Previous investigators have considered them non-pathogenic for mice.

TABLE 3

Serological characteristics of facultative, sporulating, aerogenic bacilli

SERA	ANTIGENS—PER CENT OF STRAINS AGGLUTINATED	
	Aerob polymyxa (71 strains)	*Aerob macerans* (16 strains)
Bacillus macerans (Schardinger)	0	100
Bacillus acetoethylicus (Northrop et al.)	0	100
Bacillus asterosporus (Bredemann)	26 76	0
Bacillus asterosporus (Bredemann)	23 94	0
Astasia asterospora (A. Meyer)	49 29	0
Bacillus aerosporus (Greer et al)	36.62	0
B. mycoides var. *ovaethylicus* (Wagner)	22 53	0
Aerobacillus polymyxa (Donker)	60 56	0
Aerobacillus polymyxa (Donker)	36 62	0
Aerobacillus polymyxa (Authors)	56 33	0
Aerobacillus polymyxa (Authors)	54 92	0

Agglutinin production. Heat-killed saline suspensions of selected strains were injected into the marginal ear vein of healthy rabbits. The cultures employed were selected as being representative of the various types described in the literature and, where possible, the original strains were utilized.

The 11 organisms selected were:

***Bacillus macerans*, original strain of Schardinger.**

Bacillus acetoethylicus*, original strain of Northrop *et al.

Bacillus asterosporus, 2 strains, supposed to be the original strains of Bredemann.
Astasia asterospora, original strain of A. Meyer.
Bacillus aerosporus, original strain of Greer *et al.*
Bacillus mycoides var. ovoaethylicus, original strain of Wagner.
Aerobacillus polymyxa, 4 strains; 2 strains (839 and 840 A.T.C.) studied by Donker, and 2 strains isolated in this laboratory.

Macroscopic agglutination tests were carried out in the usual way with suspensions prepared with 0.4 per cent c.p. NaCl and 0.2 per cent formaldehyde in distilled water. The results are summarized in table 3.

The agglutination reactions served to divide the cultures into two groups. The "*macerans*," or V.P. negative, high temperature group was found to be antigenically homogenous, but the "*polymyxa*," or V.P. positive, low temperature group proved to be serologically heterogeneous. Although nine "*polymyxa*" sera were prepared, a small number of strains were not agglutinated by any of their sera. Each serum agglutinated its homologous organism and a number of others, but no one serum agglutinated more than about 60 per cent of the "*polymyxa*" strains.

SUMMARY

A study of the group of facultative spore-bearing bacteria which ferment carbohydrates with the production of gas has been made for the purpose of determining their systematic relationships. Eighty-seven strains, which had been isolated from such varied sources as decaying and canned vegetables, water, soil, feces, eggs and grains, were employed. Included in this number were all available organisms of this group which have been isolated and reported in the literature, e.g., the original strains of A. Meyer, Bredemann, Wagner, Schardinger and Northrop.

From the results of this study it seems that the facultative, sporulating, aerogenic bacteria fall naturally into two groups: The "*macerans*" group, of which *Bacillus macerans* Schardinger is typical; and the "*polymyxa*" group, of which the organism isolated by Meyer (1892) and named *Astasia asterospora* is the

oldest representative extant. The reactions which differentiate the "*macerans*" and "*polymyxa*" groups are indicated in table 4.

From the results obtained in this study it seems that there are two distinct species in the group of facultative sporulating aerogenic bacteria, and if the genus *Aerobacillus* Donker is to be adopted, the specific names *Aerobacillus polymyxa* and *Aerobacillus macerans* are suggested.

The genus *Aerobacillus* should include the spore-forming rods which grow aerobically and anaerobically, produce catalase, and decompose carbohydrates with the production of acid and gas

TABLE 4

Differential characteristics in facultative, sporulating, aerogenic bacilli

CHARACTER	PER CENT POSITIVE REACTIONS	
	Aerob. macerans group (16 strains)	*Aerob. polymyxa* group (71 strains
Growth at 42–45°C. (48 hours)	100	0
Growth at 13–20°C. (1 week)	0	100
Acid and Gas in Sorbitol (72 hours, 37°C.)	100	0
Acid and Gas in Rhamnose (48 hours, 37°C.)	100	0
Voges-Proskauer reaction (72 hours, 37°C.)	0	100
Gelatin liquefaction (96 hours, 37°C.)	0	88.8
Milk coagulated (72 hours, 37°C.)	0	84.5
Agglutinated by *Aerob. macerans* serum (2 sera tested)	100	0
Agglutinated by *Aerob. polymyxa* serum (9 sera tested)	0	*

* The 9 sera show that the group is very heterogeneous serologically. Each tested serum agglutinated its specific organism and a number of other strains, but no one serum agglutinated the entire group.

in the standard fermentation tube. The synonomy of the species and the characteristics for differentiation are listed below.

1. *Aerobacillus polymyxa* (Prazmowski) Donker 1926
   Syn: *Clostridium polymyxa* Prazmowski 1880
   Granulobacter polymyxa Beijerinck 1893
   Bacillus polymyxa Beijerinck and den Dooren de Jong 1923
   Astasia asterospora Meyer 1892
   Bacillus asterosporus (Meyer) Migula 1900
   Bacillus mycoides var. ovoaethylicus Wagner 1916
   Bacillus aerosporus Greer 1928

Voges-Proskauer reaction positive; neither acid nor gas produced from rhamnose and sorbitol; no growth at 42° to 45°C., but good growth at 20°C. and slow growth at 13°C.

2. *Aerobacillus macerans* (Schardinger) Donker 1926
Syn: *Bacillus macerans* Schardinger 1905
Bacillus acetoethylicus Northrop 1919
Aerobacillus acetoethylicus (Northrop) Donker 1926

Voges-Proskauer reaction negative; acid and gas produced from rhamnose and sorbitol; good growth at 42° to 45°C. but little or no growth at 20°C.

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ACID PRODUCTION BY THE ESCHERICHIA- AEROBACTER GROUP OF BACTERIA AS INDICATED BY DISSOLVED METALLIC IRON[1]

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Received for publication July 17, 1936

The present study had its origin in certain experiments carried on in our laboratory to determine the possible influence of metals on bacterial growth. It was observed that members of the *Escherichia-Aerobacter* group when cultivated in an unbuffered glucose broth containing iron filings produced sufficient acid to dissolve some of the iron. The dissolved iron was detected by means of the potassium ferrocyanide test.

Since more acid is produced by *Escherichia coli* than by *Aerobacter aerogenes* from the same quantity of glucose, it was thought that it should be possible to add to the medium sufficient buffer to neutralize the acid produced by *Aerobacter aerogenes*, while that produced by *Escherichia coli*, under the same conditions, would still be sufficient to dissolve the iron. The investigation of this hypothesis was the purpose of the study here reported. A preliminary report has been published in abstract form (Syrocki and Fuller, 1935).

PROCEDURE

The organisms employed were isolated in this laboratory. All were Gram-negative rods which fermented lactose with gas production within 24 hours. Ruchhoft's (1931) tryptophane suspension method of purification was employed in the isolation

[1] The method employed in this study was developed by the senior author as his thesis for the Master of Science degree at the Massachusetts State College. This paper is published as contribution No. 242 of the Massachusetts Agricultural Experiment Station.

of all cultures. There were 30 strains isolated from human feces and classified as *Escherichia coli*, 30 from water classified as *Aerobacter aerogenes*, and 176 miscellaneous strains from water and from fisheries products, some of which were classified as *Escherichia coli*, some as *Aerobacter aerogenes*, and some as intermediates of the group.

Organisms were classified on the following basis:

	VOGES-PROSKAUER	METHYL RED	SODIUM CITRATE	URIC ACID
Escherichia coli	−	+	−	−
Aerobacter aerogenes	+	−	+	+

Organisms which gave results for these tests not in agreement with this classification were listed as intermediates. All media were made and tests were conducted as directed in the Standard Methods of Water Analysis (7th edition, 1933).

For the experiments following, the formula in the Standard Methods for the Clark and Lubs medium for the methyl-red and Voges-Proskauer tests was used, except that the buffer concentration was varied to fit the conditions of the experiments. These variations will be indicated in the text and tables.

Clark and Lubs did not adjust the initial reaction of their medium, but in our experiments we found that in order to avoid uncontrollable variations in results it was necessary to adjust the initial reaction of the medium to pH 7. The medium was tubed in 10-ml. portions, and 200 mgm. of degreased iron filings were added to each tube. The tubes were sterilized at 10 pounds pressure for 20 minutes. For inoculation, 24-hour cultures of the organisms in nutrient broth were used.

After the tubes had been inoculated and incubated for the desired length of time at 37°C., a few drops of a 2 per cent solution of potassium ferricyanide, $K_3Fe(CN)_6$, were added to each tube. Dissolved iron was indicated by a blue color. Tests were allowed to stand for 30 minutes before they were read.

Both potassium ferrocyanide and potassium ferricyanide solutions were tried as indicators of dissolved iron, and it was found that the ferricyanide solution gave better results than did the

ferrocyanide. This would indicate that the dissolved iron was in the ferrous state.

BUFFER CONCENTRATION

In order to determine the amount of buffer necessary to differentiate *Escherichia coli* from *Aerobacter aerogenes*, the following experiment was set up:

Four series of tubes of the medium were prepared containing 0.2, 0.3, 0.4, and 0.5 per cent respectively of buffer (K_2HPO_4).

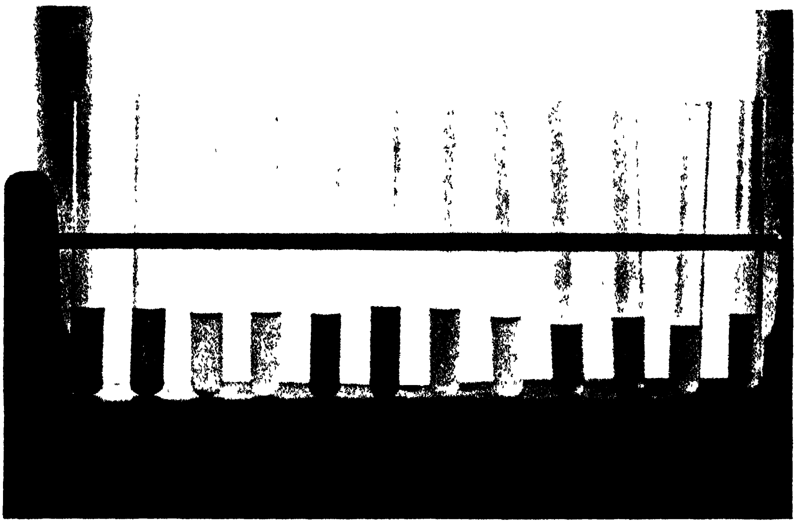

FIG. 1. SHOWING POSITIVE AND NEGATIVE TESTS FOR DISSOLVED IRON

Reading from left to right, tubes 1 to 4 contain 0.3 per cent buffer, tubes 5 to 8 contain 0.4 per cent buffer, and tubes 9 to 12 contain 0.5 per cent buffer. The two tubes at the left in each group of four are cultures of *Escherichia coli*, and the other two tubes of each group are cultures of *Aerobacter aerogenes*.

Ten strains of *Escherichia coli* and ten of *Aerobacter aerogenes* were inoculated separately into tubes containing each of the buffer concentrations. Tests for dissolved iron were made for each strain at 24, 48, and 72 hours.

This experiment indicated that 0.3 per cent buffer in the medium gave the best differentiation of *Escherichia coli* from *Aerobacter aerogenes*. Differentiation was also effected with 0.4

per cent buffer, but a less dense color and a lighter precipitate were obtained in the *Escherichia coli* cultures. Figure 1 shows some examples of the tests with different buffer concentrations, after 48-hour incubation. Best readings were obtained when the tests were allowed to stand for a half hour or longer after the addition of the ferricyanide solution, to permit the precipitate to settle out.

At 24-hour incubation some of the cultures of *Aerobacter aerogenes* gave a green color upon the addition of potassium ferricyanide, while at 48 hours the readings were more clean cut. However, it was possible to differentiate the organisms at the end of 24 hours, because the slight green observed in some of the *Aerobacter aerogenes* cultures did not confuse the readings.

THE pH AT WHICH IRON GOES INTO SOLUTION

In order to determine the pH at which the iron goes into solution, a number of tubes of the sterile iron-containing medium were taken, and 2.5 per cent sterile acetic acid was added to them in quantities sufficient to give pH values of from 4.5 to 6.0, at intervals of 0.1 pH. When the end-point had been approximately determined the pH range on either side of it was investigated at closer intervals electrometrically.

The sterile medium was incubated for 48 hours and tests were made for dissolved iron. Above pH 5.14 no iron was dissolved. at pH 5.05 the tests were + − ; at pH 4.97 to 4.99 the tests ranged from ++ to +. It appeared that the end-point of the positive test was rather sharp, and could be placed at about pH 4.98. Acetic acid was employed in this experiment because it is a predominating acid resulting from the fermentation of glucose by the organisms under consideration.

A question arose as to why the methyl-red test medium requires 0.5 per cent of buffer for differentiation between *Escherichia coli* and *Aerobacter aerogenes*, while the procedure here employed required only 0.3 per cent of buffer. To answer this the following experiment was set up: A number of strains of *Escherichia coli* were inoculated separately into a series of tubes of the medium containing metallic iron, and with buffer concentrations ranging

from 0.2 to 0.6 per cent inclusive. A second set of inoculations were made of the same organisms into the medium with the same buffer concentrations, but without the iron. After 48 hours of incubation at 37°C., pH determinations were made on both the medium with and without iron.

It was observed that the pH was higher in the medium containing iron than in the same medium without iron, in the buffer concentrations of from 0.3 to 0.6 per cent. With the 0.3 per cent buffer concentration the pH values in the iron-containing medium were at, or slightly over, the critical pH for the iron test, and were near the pH of the medium without iron and with 0.5 per cent buffer. The results indicated that a portion of the acid produced in the iron-containing medium was neutralized or inactivated, probably by being combined with the iron.

APPLICATION OF THE REACTION

One entire series of 236 cultures of the *Escherichia-Aerobacter* group were taken for a study of the application of the test. These included 30 stock cultures each of *Escherichia coli* and *Aerobacter aerogenes* which had been carried in the laboratory and frequently retested for their purity, for over two years. A comprehensive study of the organisms was made in which the Voges-Proskauer, methyl-red, sodium citrate, and uric acid tests were repeated, and the reaction of the organisms to the dissolved-iron test was determined. The results are summarized in table 1.

The results indicated that by the use of the dissolved-iron reaction, *Escherichia coli*, except for one strain, was sharply differentiated from other members of the group. The same was true of the 5 *Citrobacter* strains (these were so classified according to the article by Werkman and Gillen, 1932). In general, the methyl-red positive tests agreed well with the positive tests for dissolved iron, except with the organisms of group XII of table 1. These represented 9 of the 11 methyl-red positive strains which gave negative tests for dissolved iron, and they were aerogenes-like in their other reactions.

A comparison of the dissolved-iron reaction with the weakly positive methyl-red test showed that 72 per cent of the organisms

which were weakly methyl-red positive were negative to the dissolved-iron test. This could be explained by the fact that the critical pH for the dissolved-iron test was lower than that for the methyl-red test.

TABLE 1

Dissolved-iron test compared with other tests with bacteria of the Escherichia-Aerobacter group

CLASSIFICATION	NUMBER OF CULTURES	VOGES-PROSKAUER	METHYL RED	Na CITRATE	URIC ACID	Fe +	Fe + −	Fe ? or −
I. *Esch. coli*	36	−	+	−	−	34	1	1
II. *Esch. coli* (?)	27	−	+ −	−	−	6	3	18
III. *Citrobacter*	5	−	+	+	−	5	0	0
IV. *Aero. aerogenes*	78	+	−	+	+	4	0	74
V. *Aero. aerogenes* (?)	3	+ −	−	+	+	2	0	1
VI. Intermediates	17	+	+ −	+	+	2	0	15
VII. Intermediates	3	+	−	*	*	1	1	1
VIII. Intermediates	9	+ −	?	+	+	0	0	9
IX. Intermediates	5	+	+	*	*	4	0	1
X. Intermediates	12	+	+ −	*	*	2	1	9
XI. Intermediates	7	+ −	+ −	*	*	1	1	5
XII. Intermediates	19	+ −	+	+	*	7	3	9
XIII. Intermediates	5	−	+ −	+	*	3	0	2
XIV. Intermediates	10	−	−	*	*	5	1	4

* Sodium citrate and uric acid reactions variable.

Summary

	NUMBER OF CULTURES	Fe +	Fe + −	Fe −
Methyl red +	65	50	4	11*
Methyl red + −	68	14	5	49
Methyl red − or ?	103	12	2	89

* 9 of the Fe − were Aerogenes-like intermediates, Voges-Proskauer + − and Na citrate +.

Of 103 strains which were methyl-red negative, 12 gave positive tests for dissolved-iron. The reason for this discrepancy is not apparent. Based on the results with the strains which were weakly methyl-red positive, all methyl-red negative strains should have given negative dissolved-iron reactions. It is worthy of note that only 4 out of 78 strains which were definitely classified

as *Aerobacter aerogenes* gave positive dissolved-iron reactions, and none of the four were from the stock cultures.

The production of acidity from carbohydrate metabolism is a characteristic more common to bacteria of the *Escherichia-Aerobacter* group than are most of their physiological activities. Michaelis and Marcora (1912) showed that a certain culture of "*Bacillus coli*" ceased activity in lactose bouillon at a hydrogen-ion concentration of 1×10^{-5} N. They considered this point to be a physiological constant of the organism. Clark (1915) made the following statement: "The final hydrogen-ion concentrations differ by such small amounts that the work here reported may be considered as a confirmation of the claim of Michaelis and Marcora that the final hydrogen-ion concentrations are a physiological constant for *Bacillus coli*."

The sensitivity of the potassium ferricyanide test for the detection of dissolved iron in the cultures has not been determined. When ferrous sulfate was used as a test solution it was found that a 1:10,000 dilution gave a definitely positive test with the ferricyanide. The dissolved iron is probably mostly combined with organic acids as ferrous salts, which are relatively unstable. This fact probably accounted for difficulties encountered in the attempts to determine the sensitivity of the test. It appeared, however, that the test was sufficiently sensitive to differentiate *Escherichia coli* from other members of the group dependably when the conditions of the experiment were standardized. Further study will be made to determine more accurately the sensitivity of the test with organic ferrous salts.

The investigation reported in the present paper represents a preliminary study of a method which may, we hope, be developed as a useful means of differentiating *Escherichia-Aerogenes* bacteria, and especially intermediates of the group, on the basis of their acid production from carbohydrates. We are interested in the procedure for the study of the biological behavior of pure cultures of the organisms, rather than as a tool to be employed in water analysis, because it is by such biological study that the source and sanitary significance of various members of the group can be better understood.

SUMMARY

1. Bacteria of the *Escherichia-Aerobacter* group were cultivated in a peptone-glucose medium containing iron filings. All organisms produced sufficient acid in 24 hours to dissolve some of the iron. Potassium ferricyanide solution was employed as an indicator of dissolved iron.

2. When 0.3 per cent buffer (K_2HPO_4) was added to the medium, *Escherichia coli* still was able to produce sufficient acid to dissolve the iron, but the acid produced by *Aerobacter aerogenes* was neutralized by the buffer and no iron was dissolved sufficient for detection with ferricyanide.

3. In the medium without iron the cultures attained pH values lower than in the medium with iron.

4. When employed with pure cultures the procedure made possible a satisfactory differentiation of *Escherichia coli* from *Aerobacter aerogenes* and from intermediates of the group. Differentiation of other members of the group from each other was not accomplished.

5. The method is suggested as a means of studying the acid production of the organisms from various carbohydrates.

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THE OCCURRENCE OF SALMONELLA, SENFTENBERG TYPE, IN A DISEASE OF TURKEYS[1]

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Received for publication August 6, 1936

In June, 1936, the writer received from Dr. J. J. Black two cultures isolated from young poults. Dr. Black stated that the livers of the turkeys from which the cultures were isolated were icteric and there was some distention of the ureters with urates. Some of the birds in the flock were affected with a derangement of the hock joint which caused the foot to turn outward. The mortality did not exceed 10 per cent of the flock.

Examination of the cultures revealed that they were composed of motile bacilli which possessed the cultural and biochemical characteristics generally attributed to the genus *Salmonella*. Glucose, trehalose, arabinose, rhamnose, xylose, sorbitol and dulcitol were fermented with the formation of acid and gas. Lactose, sucrose and inositol were not attacked. Hydrogen sulfide was formed and there was a prompt production of acid in tartrate agar.

Agglutination tests with alcohol-treated suspensions showed that the organisms were agglutinated to one-half the titer of a serum for the London type and to the full titer of a serum derived from the Senftenberg type. Absorption tests revealed that the organisms were capable of effecting a complete removal of somatic agglutinins from Senftenberg serum but that they left a considerable residue of unabsorbed agglutinins for *Salmonella*

[1] The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

anatum and the London type in London serum. The organisms from poults contained the somatic factors I and III.

Tests for floccular antigens with serums containing agglutinins for the various group factors of the Kauffmann-White classification failed to indicate the presence of a group phase. The only flocculation observed was in Senftenberg serum and serums for the varieties of *Salmonella enteritidis*. The organisms in question were agglutinated to 20 per cent of the titer of serums of the Jena and Dublin varieties of *S. enteritidis* but were unable to remove agglutinins for the homologous strains. The turkey strains were agglutinated to the titer of a Senftenberg serum and were able to cause a total exhaustion of agglutinins for the homologous strain. These results indicate that the cultures contained the specific factors g and s.

From the results given above it is obvious that the bacilli from poults are monophasic Salmonellas having the antigenic formula I, III: gs:—. The cultures are serologically identical with the Senftenberg type of Kauffmann (1929) and the Newcastle type of Warren and Scott (1930). Kauffmann and Mitsui (1930) demonstrated that the only difference in these types is the production of hydrogen sulfide by the Senftenberg type and the failure of the Newcastle type to produce this substance. Since the organisms from poults produced hydrogen sulfide they must be classified as members of the Senftenberg type.

As Kauffmann (1931) pointed out, the Senftenberg-Newcastle type has not been proved to cause human disease. The strain isolated by Warren and Scott was derived from the stool of a healthy woman. One strain studied by Kauffmann (1930) was associated with the Berlin type in a case of gastro-enteritis. The second culture of this type which he studied was isolated from the stool of a person whose physical condition was unknown.

The identification of the cultures from turkeys as members of the Senftenberg type not only constitutes the first recognition of this species in animals, but is also the first record in which this organism alone was definitely associated with a pathological condition.

SUMMARY

Two cultures isolated from a disease of young turkeys were demonstrated to be members of the Senftenberg type. This report constitutes the first recognition of this type in animal disease.

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A REACTION WITH IRON COMPOUNDS FOR THE DETERMINATION OF B. ANTHRACIS AND OF ITS PATHOGENICITY

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Received for publication, August 6, 1936

Well known as is anthrax and its causative agent, *Bacillus anthracis*, there are many important facts about the disease and the organism that are very confusing. The mechanisms by which the anthrax bacillus causes the death of an infected animal are unknown, and the relationship of this organism to other bacteria resembling it is far from being clear. Many properties stated as being characteristic of *B. anthracis* are now known to be merely common cultural and morphological characters. Many times the only criteria for differentiating the anthrax bacillus from other spore-bearing aerobic bacilli resembling it are the pathogenicity tests, and these, although suitable for clinical tests on typical strains of *B. anthracis*, are for purely bacteriological purposes, useless in cases where the organism has been attenuated in some way by artificial treatment. Also, animal inoculation tests are always attended with danger and extreme care must be exercised whenever they are performed. The Ascoli thermo-precipitin test has been of considerable practical value in post-mortem diagnosis of anthrax, but pseudo-anthrax bacilli give the test at times, thereby making it not absolutely specific. Other confusing phenomena associated with this group of organisms led to this present study.

The reaction to be described in this paper was developed primarily for a simple differentiation between *Bacillus anthracis* and *Bacillus subtilis*, although unforeseen possibilities, to be discussed later, became evident. The type of reaction is new and original and its application to the study of other bacteria may be

of considerable value. The study was made on a collection of cultures furnished by various institutions.[1] Some of these cultures labelled *B. anthracis* proved to be other organisms. Since many schools had entirely different ways of labeling their cultures, a simplified classification has been adopted to suit the purpose of the present investigation. The collection consisted of the following microörganisms: 18 strains of virulent *B. anthracis*, 8 strains of *B. anthracis* which were non-lethal to white mice when 24-hour broth cultures in doses of 0.25 cc. were injected subcutaneously, 12 cultures of *Bacillus subtilis*, 3 of *Bacillus cereus*, 2 of *Bacillus mesentericus* and 2 unidentified spore-bearing bacilli.

There are two methods for performing the test to be described, the direct and indirect, both of which give positive and negative results. Virulent strains of *B. anthracis* give positive direct and indirect reactions, avirulent strains (*B. anthracis* which fail to kill white mice) give a negative direct reaction and a positive indirect reaction, *B. subtilis* and other sporing bacilli studied give negative direct and indirect reactions.

METHODS AND DESCRIPTIONS OF THE DIRECT POSITIVE REACTION

To obtain these reactions two similar methods are used, the first of which, the direct method and interpretations, is considered. The reagent used is a 10 per cent aqueous solution of ferric chloride, the medium, a 3 inch but-less slant of Bacto-North Gelatine Agar. The formula of this dehydrated North Gelatine Agar is that of R. S. Spray, and is manufactured by Difco, Detroit.

[1] The author wishes to express his sincere thanks to the following persons for their contributions of the bacterial cultures used in this study: Dr. Gerber of the City Laboratories of New York, Prof. Day of Northwestern University Medical School, Prof. Cowles of Yale University Medical School, Dr. Gold of Chester, Pa., Prof. Henrici of Minnesota University Medical School, Miss Grambell of The University of Chicago, Dr. Williams of the U. S. P. H., Washington, D. C., Dr. McKinley and Dr. Hanks of George Washington University Medical School.

The author also wishes to thank Dr. Spray, Dr. Stanley and Mr. Hawk who helped in various technical problems. These men are all members of the West Virginia University School of Medicine.

Each liter contains:

Infusion from	500 grams veal
Bacto-peptone	20 grams
Bacto-gelatine	20 grams
Soluble starch, Difco	10 grams
Sodium caseinate, Difco	2 grams
Sodium chloride, C.P.	5 grams
Bacto-agar	15 grams

In preparing it, dissolve 77 grams per 1000 cc. distilled water by boiling for a few minutes, tube and sterilize for 15 or 20 minutes at 15 pounds pressure (120°C.). The final pH is 7.4 ±.

B. anthracis is inoculated on a slant of North Gelatine Agar so that it may grow uniformly over the entire surface. The tube is incubated at 37°C. and when such a growth is obtained (which may require less than nine hours), 0.5 cc. of 10 per cent ferric chloride solution is delivered, by means of a pipette or dropper, to the bottom of the tube so that the chloride does not touch the bacteria on the upper portion of the slant. The tube is then placed in an upright position and re-incubated at 37°C. It may require only a few hours to obtain a growth sufficiently abundant to permit the introduction of the ferric chloride into the tube without retarding the growth of the organism. When a luxuriant growth is obtained ferric chloride does not hinder further growth. The optimum temperature is 37°C. but satisfactory results were gotten at room temperatures of about 30°C. One-half cubic centimeter of ferric chloride solution was found to be the optimum quantity. Too small or large quantities are disadvantageous because they interfere with specific diffusion and do not permit ideal division of the slant into the zones or bands to be described.

Shortly after the chloride has been introduced into the tube, two major horizontal zones develop in the medium, an upper and a lower. The lower one is the same in all types of reactions and is therefore of little importance; it contains the ferric chloride and is dark, due to the formation of various iron products. The important upper zone is light and unchanged at first. After several hours there is noticed a reddening of this zone, the reddening becomes more intense and within 24 hours, if the ferric chloride has been introduced early, the reaction will be very distinct. At this stage, when the slant is viewed from behind by reflected

light and from the side (in the strong reaction) by transmitted light, the two zones will stand out clearly. The upper one is of a purple-red color; this color which is at first concentrated in the lower half of the upper band later extends to the top of the slant. The lower zone will be an orange red in color because of the iron compounds. These two bands are separated by a narrow clear area. When the surface of the slant is examined the zones are not so well appreciated; here, the upper band is separated from the lower by a white line. Later the clear intermediary area is lost and the lower and upper zones meet. The white line seen on the surface becomes darkened. This dark line is clear cut and has never been seen to spread upward. Other transformations are noticed in this active area between the two major zones but are irregular and of no apparent significance or importance. The reddening of the upper zone, which may be of varying intensities, will be referred to elsewhere.

A word of caution should be given here. It is true that such potent strains of *B. anthracis* as are naturally met with will give a distinct reaction which can not be mistaken, but great care must be taken in making readings of extremely weak strains that may be encountered in very old or attenuated cultures. In these cases, indirect methods should also be used. Most reliable readings in such reactions are those obtained 24 hours after the ferric chloride has been added to the bottom of the tube.

The production of the red upper stratum is the direct positive reaction, which is always given by virulent strains of *B. anthracis* and has been seen as early as 18 hours. A negative reaction is one in which the upper zone remains unchanged, that is, does not become red.

METHODS AND DESCRIPTION OF THE INDIRECT POSITIVE REACTION

Early in this study it was noted that *B. subtilis*, when inoculated with *B. anthracis*, inhibited the reaction (but not the growth) given by the anthrax bacillus. Yet, strains of *B. anthracis*, non-lethal to white mice and which failed to give the reaction did not inhibit the positive reaction given by virulent strains of *B. anthracis*. This led to the development of the indirect reaction.

The procedure for carrying out this type of reaction is the same as the direct method except that the slant is inoculated with the microörganism in question and with a strain of *B. anthracis* which grows fairly well and that gives a strong direct positive reaction. The strain of *B. anthracis* to be used should be carefully selected by results obtained in routine tests with known virulent and avirulent strains of *B. anthracis*; with *B. subtilis* and other spore-bearing aerobic bacilli. The ferric chloride is added only after a 24-hour growth is present. In the indirect tests, controls of the anthrax bacillus and of the organism in question should be run, since the lack of growth of the unknown bacterium can not inhibit a positive reaction. Indirect reactions

TABLE 1

Results of the test

ORGANISM	NUMBER OF STRAINS	DIRECT REACTION	INDIRECT REACTION
B. anthracis:			
Virulent	18	+	+
Avirulent	8	–	+
B. subtilis	12	–	–
B. cereus	3	–	–
B. mesentericus	2	–	–
Undetermined bacilli (spore-bearing aerobes)	2	–	–

obtained will be reliable only if there is an abundant growth of the unknown organism.

A positive indirect reaction has the same characteristics as the positive direct reaction. The reaction, which should be of maximum intensity, is given by both virulent and avirulent strains of *B. anthracis*. All other bacteria so far tested have given negative reactions (*vide* table 1), but some of these organisms, for lack of growth, may fail to inhibit the positive reaction. There is, however, another phenomenon described below, which the author has called surface smearing of the upper zone. When this is seen, and is especially noticeable 24 hours after the ferric chloride has been added, the unknown organism is not *B. anthracis* even though

there is an indirect positive reaction. It is evident that the negative reaction is a final finding. In the indirect method an incubation temperature of about 30°C. yields better results than a temperature of 37°C.

Results on *B. cereus*, *B. mesentericus*, etc., merely suggest that these and all other allied bacteria would be negative.

TABLE 2

Correlation of pathogenicity and strength of reaction

STRAIN OF B. ANTHRACIS		MICE DIED IN	STRENGTH OF REACTION
Pathogenic	1c*	22 hours	++++
	2c	22 hours	++++
	3c	24 hours	++++
	4	24 hours	++++
	5c	25 hours	++++
	9c	36 hours	+++
	16	48 hours	++
	17	52 hours	++
	18	53 hours	+
Non-lethal	1		–
	2		–
	3		–

White mice were subcutaneously injected with 0.25 cc. of 24-hour old broth cultures.

* The letter (c) post-fixed after the strain number signifies that the organism was isolated from a clinical case of anthrax. The other cultures were either isolated from contaminated shaving brushes and imported sheep wool or the original source is unknown.

For indirect reactions virulent strain *B. anthracis* 5c (*vide* table 2) was used.

NEGATIVE REACTION

Negative reactions given by avirulent strains of *B. anthracis* are, except for the unchanged upper zone, identical with the positive reactions. However, all available cultures of *B. subtilis* and other sporing aerobic bacilli tested (many more of these should be studied) usually show another characteristic change. The upper zone is negative but when the surface of the slant is examined,

the dark line of demarcation of the upper and lower zones does not remain confined like the corresponding dark line of the anthrax reaction. In this case the upper border is not clear cut but the muddy-looking iron compounds, tend to gradually diffuse more and more over the lower portion of the surface of the upper zone. This change, which has been called surface smearing, has been seen in all organisms other than *B. anthracis*. Sometimes this phenomenon occurs quite early but one of the spore-bearing bacillus does not show it until quite late. Only one strain of avirulent *B. anthracis* shows (by direct method) this phenomenon very late. However, it is in this case abolished by the indirect procedure. Unless this surface smearing is pronounced, too much importance should not be given to it.

Negative direct reactions are given by avirulent strains of *B. anthracis* and by *B. subtilis* and similar organisms. Negative indirect reactions appear the same as in the former case and are given only by *B. subtilis* and other allied organisms (*vide* table 1).

RELATION TO PATHOGENICITY

It was fortunate that the first organisms investigated were virulent, and gave strong positive reactions. Later in the course of studies, some strains of *B. anthracis* were introduced which did not give the reaction. These results seemed to indicate failure and for a time the investigation was halted. Meanwhile some new cultures were received, which all gave strongly positive results. The history of these cultures showed that they had been isolated from rather recent clinical cases of anthrax. In a study of the history of the negative-reacting organisms, it was discovered that some of them were vaccine strains that had been attenuated for long periods at 42°C. At this time quite a number of cultures had been collected and in testing these by the direct method, it was found that they gave the positive reaction in varying intensities. Pathogenicity tests on white mice were run and cultures giving strongly positive reactions, always killed white mice in about 24 hours; those that gave negative reactions failed to kill. Strains that were lethal to mice in 2 days gave reactions that were weaker than more virulent cultures and

stronger than less virulent cultures that killed on the third day (*vide* table 2). Examination for spores in these cultures showed them to be abundantly formed in the avirulent strains as well as in the weakly and strongly potent ones.

The cause of this interesting correlation is not known; it may be a mere coincidence or it may be closely associated with the pathogenic properties that permit the organism to cause its lethal effects on an infected animal. Gozony and Kramar (1922) showed that the more virulent the strain of *B. anthracis* the greater was its power of reducing methylene blue.

Bouchard (1889) first showed that if the cultures of *Pseudomana pyocyaneas* were injected into guinea pigs and rabbits after they had been inoculated with anthrax cultures or blood, the animals were saved. This work was later confirmed by Emmerich and Löw (1899) who ascribed the effect to an enzyme which they called "pyocyanase." With various staphylococci, Frank (1899) observed similar results. Findings of Silberschmidt and Schoch showed *Eberthella typhi, Escheirchia coli,* Friedlander's bacillus and *Pseudomonas pyocyaneus* to be antagonistic in guinea pigs and mice if injected simultaneously with *B. anthracis.* Sanarelli and Gudel (1927) substantiated the statements of Silberschmidt and Schoch on the antagonism of *Escheirchia coli* to *B. anthracis.* Using these same organisms and carrying out the indirect procedure, it was found that they inhibited the reactions of the extremely virulent cultures of *B. anthracis. P. pyocyaneus* also antagonized the growth of the anthrax bacillus.

CHEMISTRY

Little of the chemistry of this reaction is known but there are some facts worth mentioning. Besides diffusion of the iron salts into the upper zone there is a true chemical combination of these salts with products formed by *B. anthracis.* Apparently pH changes do not determine the reaction. Such facts as distinct coloration (different from that produced by simple diffusion of ferric chloride and other iron salts), and inhibition of the reaction by *B. subtilis* and not by avirulent *B. anthracis* as in the case of the indirect reaction, make it probable that the change is due to some substance abundantly produced by virulent organisms and

less abundantly produced by avirulent organisms. Investigation of the two major zones reveals that ferric and ferrous ions exist in both of them. Ferrous sulfate has been used in place of the chloride and although it gave a more intense reddening in the upper zone it was not as satisfactory as the ferric chloride for many reasons. When ferrous sulfate is used, both ferric and ferrous ions exist in the major zones. Hydrochloric acid removes the red color of the upper band; small quantities of sodium hydroxide have very little effect on it. The exact nature of this substance, which seems to be derived from the gelatin portion of the medium, is still unknown. It and its combination with iron seem to be only moderately stable. The reason for the diffusion of the iron ions from the lower zone into the upper one cannot be given at present, but the ions are probably chemically attracted into that zone by this substance with which they unite.

If ferric chloride is poured over the slant's surface, in the case where the *B. anthracis* would give a strong reaction, a red coloration will be produced. This adds weight to the view that the color is due to a compound formed with iron. This method can not be used in practise since weak reactions are masked by the color of the by-products of the ferric chloride.

DISCUSSION

This reaction is introduced not only as an identification reaction for *B. anthracis* but also as a specific example of a new method for studying the chemistry of other bacteria since it incorporates advantages that other types of reactions lack. The following are interesting points to note: (1) the reaction introduces the factor of specific diffusion, (2) it permits the use of a reagent that would mask any coloration produced were the reagent used in any other way, (3) the method permits the growth of bacteria in the presence of a toxic substance, (4) the indirect type of reaction described above may also be used, (5) the very definite correlation of the strength of the positive reaction with the virulence of the organism may have considerable importance in the study of the mechanisms by which *B. anthracis* produces its lethal effects.

Some 30 different bacteria have been tested and *B. anthracis*

was the only bacterium that gave the positive reactions. Not enough strains of these and other organisms have been tried to be able to say that the anthrax bacillus is the only organism capable of reacting as it does.

It would be interesting to know the effects of repeated injections of cultures of *B. subtilis* on an animal already inoculated with a virulent strain of *B. anthracis*. The effects of administered iron salts on such an animal would be equally interesting.

SUMMARY

1. The paper describes a new type of chemical test for the identification of *Bacillus anthracis*.

2. There are two procedures for obtaining the reaction, the direct and the indirect, and both give positive and negative results. Virulent cultures of *B. anthracis* give positive direct and indirect reactions, avirulent strains of *B. anthracis* give negative direct and positive indirect reactions, *Bacillus subtilis* and other spore-bearing bacilli tested give negative direct and indirect reactions.

3. There is a definite relationship between the strength of the reaction and the potency of the strains of *B. anthracis*.

4. Little of the chemistry is known, but the fact is definitely established that a union of iron ions, whether ferric, ferrous or both, with a product produced by the *B. anthracis* takes place. The substance seems to be derived from the gelatin portion of the medium.

5. Out of some 30 different organisms tested, only *B. anthracis* has been found to give the reaction.

6. Some bacteria which prevent anthrax when they are simultaneously injected into rabbits, guinea pigs, and mice with *B. anthracis* have also shown similar antagonistic action on this reaction.

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GROWTH FACTORS FOR BACTERIA

III. SOME NUTRITIVE REQUIREMENTS OF LACTOBACILLUS DELBRÜCKII[1]

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Received for publication August 4, 1936

The existence of substances which stimulate the growth of microörganisms when added to culture media in small amounts has been known for many years. Since the pioneer work of Wildiers (1901) with yeast, and of Bertrand (1904) with bacteria, a great many microörganisms have been reported to require for growth certain "stimulants" present in extracts of plant and animal tissue. An adequate review of the earlier literature on this subject is given by Peskett (1933), while most of the later literature has been reviewed by Sayhun and collaborators (1936).

In a previous paper Wood, Tatum and Peterson (1936) reported two factors necessary for vigorous growth of certain propionic acid bacteria in a synthetic medium. Both factors are present in yeast extract. One was prepared from a water extract of potato, the other was obtained from corn. Since the propionic acid bacteria are generally considered to be closely related to the *Lactobacillus* group, an investigation of the requirements of certain of the latter organisms was begun. This paper deals with sources and properties of certain factors essential for vigorous growth of a representative organism of the group.

A water extract of potato was very effective in stimulating growth of *Lactobacillus delbrückii* in a peptone medium; peptone itself contained a factor necessary for growth in acid hydrolyzed media; and both factors could be obtained from a water extract

[1] Supported in part by a grant from the Special Research Fund of the Graduate School.

of liver. These substances have been partially purified, and some of their properties determined.

EXPERIMENTAL

Cultures and media. Pure cultures of the following micro-organisms were used in this investigation: *Lactobacillus delbrückii*, culture 3; *Streptococcus lactis*, culture R; and *Lactobacillus pentoaceticus*, culture 41-11. Cultures of *L. delbrückii* were incubated at 37°C.; the other cultures at 28°C.

In all cases the basal medium contained 1 per cent glucose, mineral salts,[2] and a suitable source of nitrogen, peptone or hydrolyzed casein. The final concentration of all components of the medium, other than those whose effect on bacterial growth it was desired to determine, was the same in all tubes of any one experiment. One per cent of inoculum from a 48-hour culture of the organisms in malt sprouts medium was used in all cases. The fermentations were carried out in test tubes $\frac{5}{8}$ inch in diameter, containing 10 cc. of medium.

Analytical methods. The amount of acid produced was determined by titration with 0.1 N sodium hydroxide using bromthymol-blue as the indicator. Acidity was expressed in cubic centimeters of 0.1 N acid produced per 10 cc. of medium. When the weight of acid produced was desired, this was calculated as lactic acid. Reducing sugar was determined by the micro-method of Stiles, Peterson and Fred (1926). Nitrogen in various extracts was determined by the micro-Kjeldahl method described by Pregl (1930), using copper selenite as the catalyst.

Selection of organism and medium. The crude potato extract used in these experiments was prepared as described by Tatum and co-workers (1934). Whole Wisconsin potatoes were washed and ground. The juice was expressed, autoclaved at 15 pounds pressure for one-half hour, and the coagulated protein filtered off. The filtrate was then concentrated and stored under toluene. For use, it was diluted so that 1 cc. was equivalent to approximately 1 gram of raw potato.

[2] The mineral salts were used in the following concentrations: K_2HPO_4, 0.50 gram; KH_2PO_4, 0.5 gram; $MgSO_4 \cdot 7H_2O$, 0.2 gram; NaCl, 0.01 gram; $FeSO_4 \cdot 7H_2O$, 0.01 gram; $MnSO_4 \cdot 3H_2O$, 0.01 gram; H_2O, 1000 cc.

The effect of this extract on certain of the lactic bacteria was determined by adding it in amounts not exceeding 3 cc. per 10 cc. of medium to media containing either 0.5 per cent peptone, 0.5 per cent malt sprouts, or 1.0 per cent corn. Tubes of these media were then inoculated with cultures of *S. lactis*, *L. delbrückii* and *L. pentoaceticus*. The amount of acid produced was determined after 8 days. The results are given in table 1. The crude extract brought about a definite increase in acid production in

TABLE 1

Effect of potato extract on production of lactic acid in various media

ORGANISM	CRUDE POTATO EXTRACT ADDED TO MEDIUM*	0.1 N ACID PRODUCED IN 8 DAYS PER 100 CC. MEDIUM		
		Malt sprouts medium	Peptone medium	Corn mash medium
	cc.	cc.	cc.	cc.
S. lactis, No. R	0	0 73	1.77	0 24
	1	1.38	2 45	1.10
	2	1.75	2 24	1 44
	3	1.88	2 74	1 28
L. delbruckii, No. 3	0	2 02	0 75	1.72
	1	4.17	3 86	3 13
	2	4 52	7.10	3.96
	3	5.58	9.25	3 95
L. pentoaceticus, No. 41-11	0	1.36	0.47	0.15
	1	2 25	0.53	1 00
	2	2 40	0.54	1 25
	3	2 51	0 67	1.10

* One cubic centimeter potato extract represented approximately 1.0 gram raw potato and contained 11.9 mgm. solids.

every case. However, the stimulation was especially striking in the case of *L. delbrückii* on the peptone medium. This organism and this medium were therefore employed in attempts at further purification of the active principle in the potato extract.

Rôle of potato extract in glucose fermentation by L. delbrückii. More than one explanation for the increased acid production noticed might be suggested. A plant extract might bring about increased acid production (1) by supplying some sugar more easily fermented than glucose; (2) by increasing the fermentation

rate of the individual cells; (3) by increasing the number of cells, each of which ferments sugar at approximately the same rate as a cell in the absence of the "stimulant;" (4) by changing the course of the fermentation, resulting in production of a greater proportion of acidic substances per unit of glucose destroyed; (5) by buffering the medium so that acid production would not slow down the rate of fermentation; or (6) by a combination of the

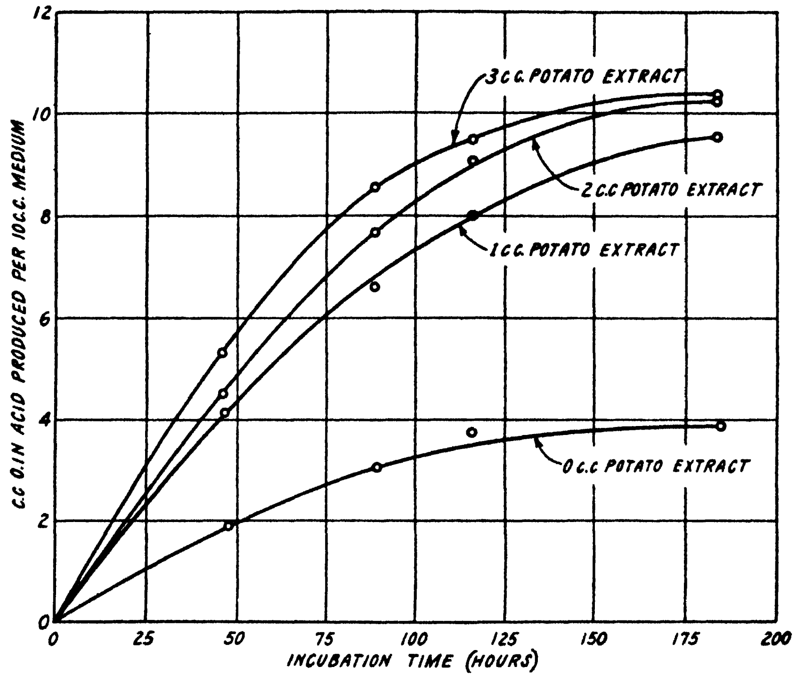

FIG. 1. STIMULATING EFFECT OF POTATO EXTRACT ON ACID PRODUCTION IN PEPTONE MEDIUM

above effects. Data indicating which of these effects are significant are given below.

A series of tubes were prepared containing different concentrations of the potato extract. Duplicate tubes were titrated at intervals from 0 to 185 hours. The results are given in figure 1. The potato extract caused an increased rate of acid production from the beginning of the fermentation. Furthermore, this

increase was not proportional to the amount of extract added, as might be expected, were it due to addition of small amounts of a substance easily fermented with production of acid.

The increased acid production was not due to an increase in the ratio of acid produced to the glucose destroyed (table 2). Assuming that only lactic acid was produced, almost perfect equivalence was obtained between acid production and glucose fermentation. This indicates that potato extract did not change the course of the normal fermentation, and that the increased production of acid was caused by increased glucose fermentation rather than by utilization of substances in the added potato extract.

TABLE 2

Dissimilation of glucose to lactic acid by L. delbrückii

POTATO EXTRACT IN 10 cc OF MEDIUM	AFTER 48 HOURS		AFTER 96 HOURS		AFTER 144 HOURS	
	Lactic acid produced	Glucose destroyed	Lactic acid produced	Glucose destroyed	Lactic acid produced	Glucose destroyed
cc.	*mgm per 10 cc.*	*mgm per 10 cc*	*mgm per 10 cc*	*mgm. per 10 cc.*	*mgm. per 10 cc.*	*mgm per 10 cc.*
0	14 3	15 1	18 9	19.7	23.4	23 7
2	63.2	66 6	74 8	76.6	84 5	82.9*
3	64 5	67 2	77.6	79 4	85.2	83 1*

Basal medium: 0.5 per cent peptone, 1 per cent cerelose, mineral salts.

* Ten cubic centimeters of medium (without potato extract) contained 84.9 mgm. glucose.

It was observed that in all tubes increased acid production was correlated with greatly increased visible growth. Direct plate counts were made to check this observation. Platings were made after a 48-hour fermentation in order to avoid loss of viability from exposure of the organisms to a high acidity. Later work has shown that these counts are minimum figures since the organisms occurred in chains which were extremely difficult to break up into the constituent cells. Results (table 3) show a 100-fold increase in bacterial numbers for a 4-fold increase in acid, a 200-fold increase for a 5-fold increase in acid. The most pronounced effect of the potato extract, therefore, was to increase bacterial numbers. Increased acid production followed as a necessary consequence.

To determine whether the potato extract was equally stimulating in a medium containing $CaCO_3$, fermentations were carried out in 8-ounce bottles containing 100 cc. of 3-per-cent glucose-peptone medium, to which an excess of sterilized $CaCO_3$ was added aseptically. Samples for analysis were removed at intervals with sterile pipettes. The data are given in table 4. The rate of fermentation was markedly accelerated by the extract

TABLE 3

Correlation between numbers of bacteria and acid production

POTATO EXTRACT ADDED	AVERAGE NUMBER PER CUBIC CENTIMETER	0 1 N ACID PER 10 CC MEDIUM
cc.		*cc*
0	182,000	0 43
1	18,875,000	1 78
2	34,500,000	2 00
3	46,570,000	2 22

Basal medium: 0.5 per cent peptone, 1 per cent cerelose, mineral salts.

TABLE 4

Effect of potato extract on glucose fermentation by L. delbrückii in presence of calcium carbonate

POTATO EXTRACT PER 10 CC. MEDIUM	GLUCOSE FERMENTED* AFTER						
	24 hours	48 hours	72 hours	96 hours	142 hours	190 hours	238 hours
cc.	*mgm.*	*mgm.*	*mgm*	*mgm.*	*mgm.*	*mgm*	*mgm*
0	27.9	76.5	124.8	145.5	175 5	204 9	229 0
1	29.7	128 4	192 1	206 4	235 6	248 6	263 3
2	33.5	147 2	208 3	229.9	243 4	259 8	270.9

Basal medium: 0.5 per cent peptone, 3.0 per cent glucose, mineral salts.
* Original medium contained 275.9 mgm. reducing sugar per 10 cc.

during the first half of the fermentation. Later, a depleted sugar supply became a limiting factor so that eventually destruction of sugar in the controls nearly reached that in the supplemented cultures. The data show clearly that the potato extract acts as a stimulant rather than as a buffer.

The initial oxidation reduction potential of a medium is often a factor in determining whether or not bacteria can initiate growth. However, potentiometric data showed that the action of the

potato extract was not due to its effect upon the oxidation-reduction potential of the medium. Although the extract lowered the oxidation potential, quantities of cysteine, cystine, or thioglycollic acid which lowered the potential in like amount were without effect upon growth of the bacteria.

In early experiments, considerable variation in growth was observed in similar experiments conducted at different times. An investigation of factors causing these variations showed that the final amount of growth may differ widely with the amount of inoculum, even though the smallest inoculum used was capable of initiating growth in the medium. Sterilization near neutral reaction often produced acidic substances, which stimulated growth of the organism to some extent. Therefore, size of inoculum and conditions of sterilization were carefully controlled in subsequent experiments.

Rôle of peptone in the growth of L. delbrückii. In an effort to develop a basal medium more nearly synthetic in character it was found that although the test organism grew well in a peptone or sodium caseinate (nutrose) medium in the presence of potato extract, acid hydrolyzates of peptone or casein were ineffective under the same conditions. The inclusion of tryptophane (ordinarily destroyed by acid hydrolysis) in these hydrolyzed media made initiation of growth possible, but in no case did growth in such media approach that in the unhydrolyzed media (see table 6). The disparity was not remedied by addition of cystine or cysteine to the medium. Evidently some substance other than tryptophane, and like tryptophane, essential for proper growth of the organism, was destroyed by the acid hydrolysis. It appears that *L. delbrückii* requires for vigorous growth unknown factors contained in potato extract and in peptone, in addition to known amino acids and a fermentable carbohydrate.

PREPARATION AND ACTIVITY OF POTATO FRACTIONS

In determining the effect of the various preparations on the growth of the test organism, the fractions were added in amounts equivalent to the original crude potato extract used in their preparation. A 0.5-per-cent peptone, 1-per-cent glucose, mineral

salts medium was used as a base. The results are presented in table 5. The fractions containing the greatest proportion of the stimulating substance are compared in figure 2. Details regarding the various treatments and conclusions therefrom follow:

1. Hydrolysis. 5.5 cc. concentrated H_2SO_4 were added to 50 cc. potato extract. Hydrolysis was carried out over night in the autoclave at 15 pounds pressure. Sulphuric acid was removed

TABLE 5

Biological activity of potato extract fractions

Cubic centimeters 0.1 N acid produced per 10 cc. medium

SUPPLEMENT TO BASAL MEDIUM	SUPPLEMENT ADDED PER 10 CC. MEDIUM*			
	0	1 cc.	2 cc.	3 cc.
Crude potato extract	1.61	6.73	8.05	8.74
Hydrolyzed potato extract	1.45	1.91	1.52	1.91
Ether extract of potato extract	1.40	3.90	5.10	6.03
Ether extract of potato extract plus sodium acetate			7 30	8.20
Residue from ether extraction	1.45	4.48	4.94	4.83
Ether extract plus residue		6.25	8.10	8 40
Ether-alcohol filtrate of potato extract		6.40	7.50	8 20
Ether-alcohol precipitate of potato extract		1.80	4.10	4.75
Lead acetate-ammonia filtrate from potato extract		4.40	5.80	6 32
Neuberg precipitate of potato extract		3.21	3 55	4.02
Neuberg filtrate of potato extract		6.30	7 92	8.60
Neuberg fractions combined		6.78	7.90	9.08

Basal medium: 0.5 per cent peptone, 1 per cent glucose and mineral salts.

* All fractions were added so as to be equivalent to the original crude potato extract.

with barium hydroxide. The stimulating substance was completely destroyed by this treatment.

2. Ether extraction. Crude potato extract was concentrated to small volume, acidified with sulphuric acid until acid to Congo red. Anhydrous calcium sulphate was mixed with the concentrated extract, and the whole allowed to dry. The resulting product was extracted continuously with ether for several days. Ether was removed from the extract, the residue was taken up in water, neutralized with sodium hydroxide, and diluted to volume. The residue from the ether extraction was boiled up with water,

filtered, neutralized, and concentrated to volume. The stimulatory substance was extracted by ether. The residue was less active than the extract.

3. Alcohol-ether precipitation. This procedure, as used for liver extract, has been described by Koehn and Elvehjem (1936). One

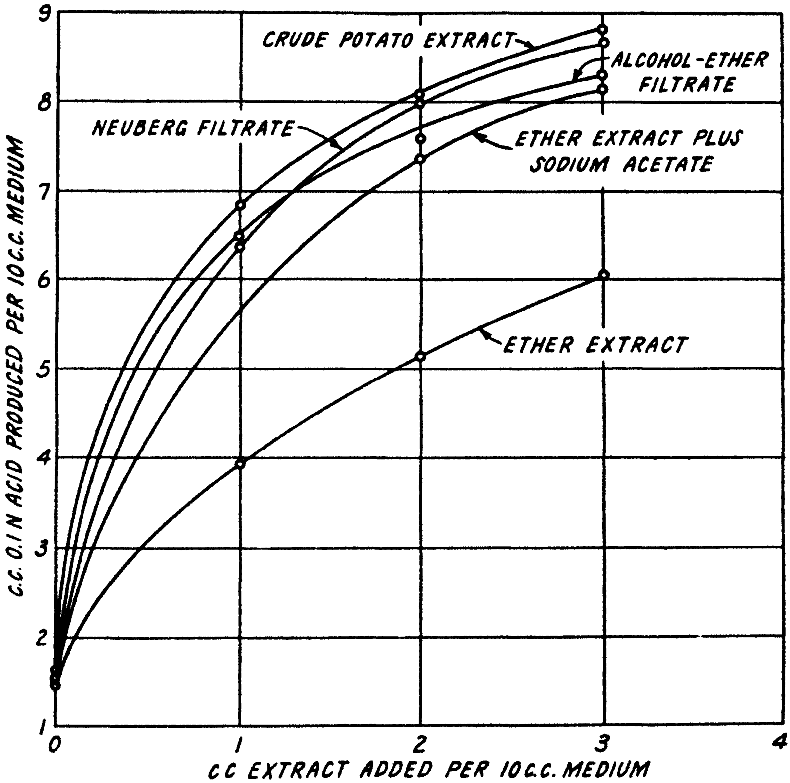

FIG. 2. STIMULATING EFFECT OF POTATO EXTRACT FRACTIONS ON ACID PRODUCTION

volume of concentrated potato extract was treated with 5 volumes of ethyl alcohol, then 6 volumes of ethyl ether were added with constant stirring. The precipitate was removed by centrifuging, washed with alcohol-ether, and dissolved in distilled water. The alcohol-ether solution was concentrated until all the organic solvents had been driven off, then diluted to volume with dis-

tilled water. The major portion of the dark-colored constituents of the extract were obtained in the insoluble fraction by this procedure. The stimulatory substance was soluble in the neutral alcohol-ether mixture.

4. Treatment with lead acetate and ammonia. Proteins, starch, sugar, and substances hydrolyzable to glucose were removed from the extract by precipitation with lead acetate in the presence of an excess of ammonia (Tatum, Peterson and Fred (1934)). The excess ammonia was boiled off from the filtrate, and the lead removed with hydrogen sulfide. Excess hydrogen sulfide was removed by boiling. The stimulatory substance was not precipitated by this treatment, and is therefore probably not related to the above substances.

5. Treatment with Neuberg's reagent. Crude potato extract was made alkaline with sodium carbonate and precipitated with mercuric acetate as described by Neuberg and Kerb (1912). The mercury was removed from filtrate and precipitate with hydrogen sulfide, and the excess hydrogen sulfide was removed by boiling. Although all proteins, peptides, and amino acids in the extract should be precipitated by this reagent, the filtrate was practically as active as the untreated extract.

Extraction of the crude potato extract with butyl alcohol by the method of Dakin (1918) was tried, but did not separate essential from non-essential substances.

When an amount of sodium acetate approximately equal to that occurring in the Neuberg filtrate was added to the ether extract, the effect of the latter became equal to that of the Neuberg filtrate, probably because of the increased buffer capacity.

The following figures show that the nitrogen content of the fractions is not a measure of their effect in stimulating growth:

Extract	Dry weight of 1 cc. mgm.	Nitrogen content of 1 cc. mgm.
Crude potato	11.98	0.775
Alcohol-ether filtrate	7.9	0.532
Alcohol-ether precipitate	3.5	0.239
Ether extract		0.049
Neuberg filtrate		0.118

From the above results, certain properties of the potato stimulant may be deduced. It is stable to autoclaving; soluble in water, ethyl alcohol, and ether. It is probably not a protein, peptide, amino acid, or a carbohydrate yielding glucose on hydrolysis. Since it is extracted by ether under acid conditions, it is not basic in character, and may be an acid of fairly simple nature. It is destroyed by prolonged acid hydrolysis.

TABLE 6

Activity of peptone fractions

Cubic centimeters 0.1 N acid produced per 10 cc. medium

SUPPLEMENT TO MEDIUM	PEPTONE EQUIVALENT OF ADDED FRACTIONS	
	0.4 per cent	0 8 per cent
None	0.75	
Hydrolyzed peptone	1.2	1 8
Peptone	7 05	8 4
Butyl alcohol extract of peptone	1 25	1 65
Residue from butyl alcohol extraction	3 63	4 05
Butyl alcohol extract plus residue	4 50	7 05
Residue from ethyl alcohol extraction of peptone	4 05	4 9
Ethyl alcohol extract of peptone	6 15	8 4

Basal medium: 0 9 per cent hydrolyzed casein, 0.01 per cent tryptophane, 1 per cent glucose, mineral salts, 0.5 cc. Neuberg filtrate of potato extract (per 10 cc. of medium).

PREPARATION AND ACTIVITY OF PEPTONE FRACTIONS

The medium used in determining which components of the peptone were necessary for growth contained, besides glucose and mineral salts, hydrolyzed casein (0.9 per cent), tryptophane, and the Neuberg filtrate of potato extract. Growth in this medium in the absence of peptone was very scant. The peptone fractions were added to this medium in amounts representing 0.4 or 0.8 per cent of the original peptone.

Results are summarized in table 6. The difference in effect of peptone and hydrolyzed peptone should be especially noted. Hydrolysis was carried out in the same manner as with potato extract.

As with potato extract, butyl alcohol extraction effected no

separation of essential constituents and seemed to destroy some of the activity. Peptone was entirely insoluble in ethyl ether so no separation could be effected with this solvent.

Ethyl alcohol extract. Extraction was made by the procedure described by Sayhun and co-workers (1936). Fifty grams of peptone were dissolved in hot water (about 35 cc.), and 250 cc. of hot 95-per-cent ethyl alcohol were added. The mixture was shaken frequently during a period of 24 hours, then allowed to stand for 24 hours longer. The clear supernatant liquid was decanted, and the extraction repeated twice, with 250-cc.-portions of 80 per cent alcohol. The alcoholic extracts were combined, acidified with 1 N sulphuric acid to pH 5.4. After standing a few hours the slight precipitate was filtered off. Almost all of the activity of the original peptone remained in the alcohol-soluble fraction. The extent of the concentration is shown by the following dry weight determinations:

	mgm per cc of 4 per cent equivalent
Original peptone	40 0
Alcoholic extract of peptone	15.4
Residue from alcoholic extraction	20 3

Since almost all of the activity was concentrated in this extract, it was used as a starting point in the preparation of all fractions other than those mentioned above. The various treatments were as follows with fermentation data given in table 7.

1. Solubility in acetone. Five cubic centimeters of the alcohol extract were treated with 25 cc. of acetone. The white flocculent precipitate was centrifuged off, washed with acetone once, and dissolved in water. The material was only slightly soluble·in approximately 80 per cent acetone.

2. Adsorption and dialysis. The active factor was adsorbed by filtering through Norite under acid, neutral, or alkaline conditions, but only a slight loss in activity was suffered on filtering through Supercel. It was readily dialyzable through a collodion membrane.

3. Treatment with lead acetate-ammonia and Neuberg's reagent. These precipitations were carried out as described above for

potato extract. The active material was precipitated almost completely by lead acetate and ammonia and by Neuberg's reagent. In the case of the latter, the slight effect of the filtrate may be ascribed to its salt content. Addition of sodium acetate and sodium chloride gave about the same effect as addition of Neuberg filtrate.

TABLE 7

Treatment of ethyl alcohol extract of peptone and activity of fractions

Cubic centimeters of 0.1 N acid produced per 10 cc. medium

SUPPLEMENT TO MEDIUM	PEPTONE EQUIVALENT OF ADDED FRACTIONS	
	0.4 per cent	0 8 per cent
Original extract	6.15	8 4
Acetone soluble	1.95	2 8
Acetone insoluble	3.8	5.2
Norite filtrate		
pH 9.0	1 45	2 40
pH 7.0	1.70	2 35
pH 6.0	1 50	2 45
Supercel filtrate pH 6.0	5 55	8.65
Collodion dialysate	6.50	7 5
Phosphotungstic acid precipitate	3 1	4 4
Phosphotungstic acid filtrate	1 5	1.8
Phosphotungstic acid fractions combined	6.25	6 9
Barium hydroxide filtrate	3.42	4 85
Lead acetate-ammonia filtrate	1 65	1.9
Lead acetate-ammonia precipitate	2 85	4 0
Neuberg filtrate	1.1	1 5
Neuberg precipitate	4 41	5.2
Neuberg fractions combined	4.3	5.4

Basal medium: 0.9 per cent hydrolyzed casein, 0.01 per cent tryptophane, 1 per cent glucose, mineral salts, 0.5 cc. Neuberg filtrate of potato extract (per 10 cc. medium).

4. Precipitation with barium hydroxide. To the neutral alcohol extract a saturated solution of barium hydroxide was added until no further precipitation took place. The solution was filtered and the excess barium hydroxide removed from the filtrate with sulphuric acid. The active material was not precipitated.

5. Phosphotungstic acid precipitation. This precipitation was

carried out in the usual manner with 30 per cent phosphotungstic acid dissolved in 5 per cent sulphuric acid. Excess sulphuric and phosphotungstic acids were removed from filtrate and precipitated with barium hydroxide. The excess of the latter was removed with sulphuric acid. Phosphotungstic acid would precipitate basic organic substances, such as any basic amino acids or peptides occurring in the peptone. The active substance was largely precipitated by this reagent.

The above properties suggest that the active substance is either a basic amino acid or a compound with properties similar to such acids.

LIVER AS A SOURCE OF ESSENTIAL GROWTH FACTORS FOR L. DELBRÜCKII

Liver and extracts of liver have long been used as sources of biological substances stimulatory to growth. Various preparations from liver were therefore obtained in order to determine whether this material could be used as a convenient source of the factors necessary for the growth of *L. delbrückii*.

Preparation of extracts. The water extract of liver used in these experiments was obtained from the Wilson Laboratories of Chicago. It is essentially a dried water extract of fresh liver from which proteins have been removed by coagulation.

The ether extract of the water soluble constituents of liver was made in the same manner as described for potato extract.

The alcohol-ether precipitate, vitamin B_2 and hepatoflavin fractions were prepared by the method of Koehn and Elvehjem (1936).[3] The preparation of the alcohol ether precipitate has already been described. The hepatoflavin fraction was removed from the alcohol-ether soluble portion of the liver extract by adsorption upon fuller's earth and subsequent elution with pyridine and methyl alcohol. The portion not adsorbed is the vitamin B_2 fraction.[4] Since the pure hepatoflavin is unstable to light, special precautions were taken with this extract to prevent its decomposition.

[3] These extracts were prepared by Dr. C. J. Koehn for his vitamin investigations and portions of them kindly supplied to the authors.

[4] The terminology used in this paper is that of Koehn and Elvehjem (1936).

Activity of liver fractions. All extracts were added to the basal medium on the basis of the dry weight of the liver extract from which they had been prepared. The effect of the crude water-extract of liver on growth in various media is shown in table 8. Although neither potato extract alone nor extracts of peptone alone are effective in producing good growth on hydrolyzed casein medium containing tryptophane, the liver extract does support such growth and must therefore contain both factors, or substances capable of replacing them. This is confirmed by the fact that the presence of potato extract in the hydrolyzed casein medium with liver extract does not increase acid production over

TABLE 8

Stimulation of growth and acid production by a water extract of liver

Cubic centimeters of 0.1 N acid produced per 10 cc. of medium

MEDIUM*	AMOUNT OF LIVER EXTRACT ADDED TO MEDIUM			
	0	0.1 mgm.	1 mgm	10 mgm
0.5 per cent peptone	0 25	0 25	2 30	6 25
0.015 per cent tryptophane	0	0	0.65	2 80
0 9 per cent hydrolyzed casein + 0.015 per cent tryptophane	0 25	0 30	4 15	9 00
0.9 per cent hydrolyzed casein + 0.015 per cent tryptophane + 1 cc. potato extract	0 75	1 30	3.00	8 75

* Also contained 1 per cent glucose and mineral salts.

that with liver extract alone except when the latter is present in very small amounts.

The distribution of the substance capable of replacing the potato extract factor was further investigated. For this purpose the effect of the different fractions of liver extract on growth of the test organism in peptone medium was determined. The results are given in table 9. The active substance was concentrated in the alcohol-ether precipitate; the vitamin B_2 fraction contained it in much smaller amounts, while the flavin fraction was entirely inactive. The activity of the vitamin B_2 fraction was much increased, however, by the addition of the flavin fraction.

The acid-ether extract of liver extract showed some stimulating properties. The substance extracted may be the same as that obtained from potato extract. This is not the main factor present, as is shown by the much greater activity of the residue from ether extraction. In the case of potato extract, the residue was less active than the ether extract. Stimulatory substances in the potato extract were also concentrated in the filtrate from the alcohol-ether precipitation, whereas in the case of liver extract they are concentrated in the precipitate. These facts indicate that factors other than those contained in potato extract and

TABLE 9

Activity of liver fractions

Cubic centimeters of 0.1 N acid produced per 10 cc. of medium

NUMBER	SUPPLEMENT TO PEPTONE MEDIUM	WEIGHT OF LIVER EXTRACT REPRESENTED BY SUPPLEMENT			
		0	5 mgm	10 mgm	30 mgm
1	Crude liver extract	0 35	5.1		
2	Acid-ether extract of liver extract	0.4	2 3	2.7	3.1
3	Residue from ether extraction	0 4	6.35	7.1	
4	Alcohol-ether precipitate fraction	0.35	5.3	6.7	7 85
	Alcohol-ether dialysate		5.25	7.55	
5	Heptoflavin fraction	0 35	0.35	0.35	0.35
6	Vitamin B_2 fraction	0.35	0.45	0.45	5.55
7	Hepatoflavin + vitamin B_2 fractions		2.85	5.6	5.4
8	Hepatoflavin + alcohol-ether precipitate		5.32	6.82	8.2
9	Vitamin B_2 + alcohol-ether precipitate		5.9	7.5	8.25
10	Vitamin B_2 + hepatoflavin + alcohol-ether precipitate		6 5	7.55	8 2

Basal Medium: 0.5 per cent peptone, 1 per cent glucose, mineral salts.

present in liver extract are capable of replacing the potato factor. An alternative explanation would be to assume that both factors are identical, but that in the case of liver extract it is in combination with a substance precipitated by alcohol-ether, from which it is not released under the acid conditions of ether extraction employed. The stimulatory substances contained in the alcohol-ether precipitate of liver extract were completely dialyzable.

DISCUSSION

The known properties of the three factors are summarized in table 10. The peptone and potato factors are evidently different. While all of the diagnostic tests have not been applied to the liver factor, it appears to contain two factors, one of which is identical with the potato factor, and another which can replace this factor. Constituents in the liver extract are also able to replace the peptone factor, which is necessary in addition to the potato factor for luxuriant growth on acid hydrolyzed media.

TABLE 10

Comparison of peptone, potato, and liver factors

PROPERTY	FACTOR		
	Peptone factor	Liver factor	Potato factor
Heat stable (pH 7.0)	+	+	+
Dialyzable	+	+	+
Solubility			
(1) Water	+	+	+
(2) 80 per cent ethyl alcohol	+	+	+
(3) Ethyl ether	−	Partially	+
Precipitated by			
(1) Alcohol-ether	+	+	−
(2) Neuberg's reagent	+		−
(3) Lead acetate-ammonia	+		−
(4) Phosphotungstic acid	+		
Adsorption on norite	+		
Destroyed by acid hydrolysis	+		+

Properties of the potato factor indicate that it is similar to the factor reported by Wood, Tatum and Peterson (1936) for the growth of certain propionic acid bacteria. Its effect on *L. delbrückii* is not correlated with its nitrogen content, and since it was extracted by ether from acid solutions, it seems to be an acid.

Kayser in 1894 showed that peptone is a very good source of nitrogen for lactic acid bacteria, especially in the presence of juice from vegetables such as onions. ZoBell and Meyer (1932), in attempting to prepare a synthetic medium which would support growth of *Brucella*, observed that in no case did known simple carbon sources in the presence of a nitrogen source support

growth nearly so well as peptone. They state "the accelerated multiplication noted from the use of peptone . . . may be due to other factors than its nitrogen content." Sayhun and co-workers (1936) obtained a preparation from the alcoholic extract of peptone which was very active in stimulating the growth of *Escherichia coli* in a synthetic medium. The factor was precipitated by phosphotungstic acid, and was destroyed by prolonged boiling with acid. Our factor is probably closely related to this factor. They reported the separation of their peptone factor into two fractions with butyl alcohol. In our case, the butyl alcohol soluble constituents of the peptone are practically without effect on the growth of *L. delbrückii.* Solubility and precipitation reactions of the factor indicate that it is a basic, nitrogenous compound of fairly low molecular weight.

The activity of liver extract in replacing potato extract was not correlated with the presence of vitamin B_2, since an extract of liver containing vitamin B_2 was comparatively inactive in stimulating growth of the test organism in a peptone medium.

SUMMARY

A water extract of potato, when added to simple media stimulated growth and acid production of a number of lactic acid bacteria. This action was most effective with *Lactobacillus delbrückii* on peptone-glucose-mineral-salts medium. The stimulating action is evident also in media buffered with $CaCO_3$, and results primarily in an increase in the number of cells.

Tryptophane is an amino acid essential for the growth of this organism. For luxuriant growth in the presence of hydrolyzed casein and tryptophane two unknown factors are necessary. One of these occurs in the Neuberg filtrate or acid-ether extract of a water extract of potato, and may be an acid of fairly low molecular weight. The other factor is basic in character, and occurs in peptone. Both are destroyed by prolonged acid hydrolysis.

Liver extract contains both of the necessary factors or factors capable of replacing them. Vitamin B_2 does not seem to play a rôle in the stimulation by liver preparations.

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GROWTH FACTORS FOR BACTERIA[1]

IV. An Acidic Ether-soluble Factor Essential for Growth of Propionic Acid Bacteria

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Received for publication August 15, 1936

The propionic acid bacteria are considered by most investigators to be fastidious in their growth requirements. Yeast extract or similar complex materials have frequently been used in media for these organisms. Recently Fromageot and Tatum (1933), and Tatum, Peterson and Fred (1936) have shown that certain plant extracts, particularly those of potato and corn, contain growth stimulants for these bacteria. Tatum, Wood and Peterson (1936a) found that ammonium nitrogen was utilized in the presence of these stimulants. They suggested that in media containing the proper growth factors ammonium nitrogen might possibly serve as the sole source of nitrogen. The present investigation is a continuation of this study. The purification of the growth factors has been undertaken with the ultimate aim of developing a medium for these bacteria in which all constituents are known. The physiology of this group could be studied far more accurately if this were accomplished.

EXPERIMENTAL

Cultures and methods

The culture used unless otherwise stated was *Propionibacterium pentosaceum* No. 11 (Hitchner (1934)). The fermentations were

[1] This work was supported in part by a grant from the Special Research Fund of the Graduate School.

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carried out in test tubes containing 10 cc. of medium, and incubated at 30°C. All substances used in the basal medium were chemically pure with the exception of the hydrolyzed casein. Speakman's inorganic salts were used in most media.[3] The media were adjusted to pH 7.0 and sterilized at 15 pounds pressure for 20 minutes. When 0.15 M phosphate buffer was used, it was sterilized separately and added aseptically to the medium.

The bacteria used for inoculation were grown in a medium consisting of 0.5 per cent glucose and 0.5 per cent yeast extract (Difco) unless otherwise stated. After 4 or 5 days' incubation the supernatant medium was removed aseptically and the cells were suspended in an equivalent amount of sterile, distilled water. Two drops of this cell suspension were used as inoculum for each tube.

The amount of growth was determined by direct titration of the acid produced with brom-thymol-blue as an indicator or by determining the glucose fermented by the method of Stiles, Peterson and Fred (1926).

Preparation of stimulant and development of medium

A medium containing Difco yeast extract, glucose and distilled water supports excellent growth of the propionic acid bacteria and apparently fulfills all their nutritional requirements. The vigorous growth obtained on this medium indicated that yeast extract would be a good source of growth stimulants. Although Tatum, Wood and Peterson (1936a) showed that these bacteria are able to utilize ammonium nitrogen the possibility was not excluded that certain amino-acids are essential. Therefore a basal medium of $(NH_4)_2SO_4$, 0.3 per cent; hydrolyzed casein, 1.5 per cent; phosphate buffer, 0.15 M, pH 7; glucose, 2 per cent, and tap water was used. Growth on the basal medium was negligible if a washed suspension of cells was used as the inoculum, but if inoculation was made directly from the yeast extract medium, some growth was obtained.

[3] The salt content of the medium was as follows: K_2HPO_4, 0.25 gram; KH_2PO_4, 0.25 gram; $MgSO_4 \cdot 7H_2O$, 0.1 gram; NaCl, 0.005 gram; $FeSO_4 \cdot 7H_2O$, 0.005 gram; and $MnSO_4 \cdot 4H_2O$, 0.005 gram; water, 1000 cc.

Numerous methods of separating active preparations from the crude yeast extract were tried and eventually it was found that a growth factor could be extracted with ether. The following method was used with uniform success. The yeast extract powder was mixed with sufficient 10 N sulfuric acid to give a thin paste, acid to Congo red. The resulting material was then taken up in an excess of plaster of Paris so that a dry and crumbly material was obtained which did not set on standing. This material was extracted continuously with ether for 48 hours. Usually a solid separated from the ether extract which consisted of succinic acid crystals and a waxy material. This inactive solid was discarded. On evaporation of the ether solution a brown sticky substance containing more succinic acid crystals was obtained. The water solution of this material was neutralized and stored under toluene at room temperature with no loss in activity. Approximately 63 mgm. of solids were obtained in the crude ether extract per gram of original yeast extract.

The effect of this ether extract on growth when added to the basal medium is shown in figure 1. One unit of yeast stimulant was chosen arbitrarily to represent the ether extract of 1 gram of yeast extract. The growth on media containing only $(NH_4)_2SO_4$ as the nitrogen source was not vigorous but with hydrolyzed casein added it approached that in a crude-yeast extract medium although there was not the same luxuriant development of cells. The increased growth in the presence of hydrolyzed casein indicated that amino acids were essential or at least beneficial. There was no growth if both ammonium sulfate and hydrolyzed casein were omitted from the basal medium. It is apparent from these results that a growth factor was present in the ether extract.

An attempt was next made to develop a medium in which approximately the optimal concentrations of the known constituents were used and in which growth could be determined by titration of acid rather than by determination of sugar destroyed. This was possible when sodium acetate was added to the medium in place of phosphate buffer. Acid production was best in the presence of 0.6 per cent sodium acetate, higher concentrations had little further effect. The ammonium sulfate concentration was

varied over a wide range and 0.3 per cent was found to be the optimum. In the presence of this concentration of ammonium sulfate, 0.9 per cent hydrolyzed casein was the optimum. From these results two basal media were selected which contained, besides glucose (1 per cent) and inorganic salts, the following constituents:

(1) *Ammonium sulfate medium*—sodium acetate, 0.6 per cent; $(NH_4)_2SO_4$, 0.3 per cent.

(2) *Hydrolyzed casein medium*—same as (1) but with 0.9 per cent hydrolyzed casein added.

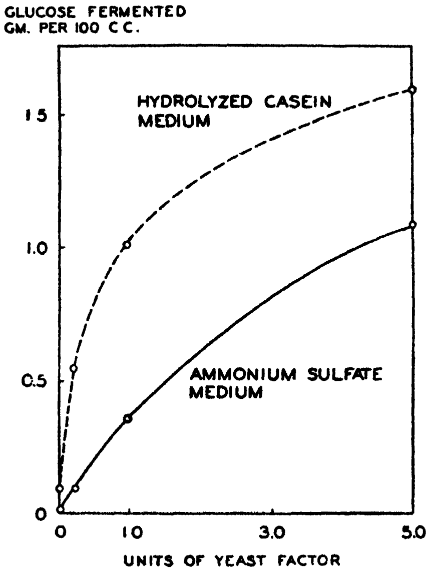

FIG. 1. EFFECT OF YEAST FACTOR ON FERMENTATION OF GLUCOSE

(Five days' fermentation. One unit equals ether extract of 1 gram crude yeast extract.)

The optimal concentration of the hydrolyzed casein was higher than should be necessary to meet the amino acid requirements of the bacteria. An explanation of this phenomenon will be offered in a subsequent paper (Tatum, Wood and Peterson (1936b)). The inorganic salt mixture used in these experiments was found to be as effective as the salts in tap water or those of yeast extract ash. Variation of salt concentration had little effect over a wide

range. No attempt has been made to establish the necessary inorganic constituents.

Typical results obtained on these media are shown in figure 2, *A*. The results are similar to those obtained with media containing phosphate buffer in which the fermented sugar was the criterion of stimulation. Sugar destruction and acid production

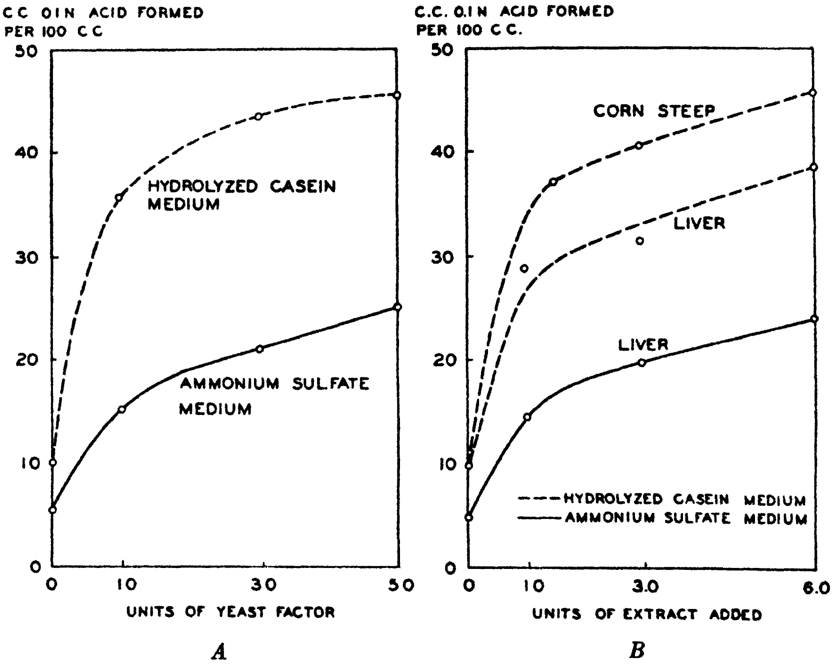

FIG. 2. *A*, effect of yeast factor on production of acid (4 days' fermentation); *B*, effect of corn-steep and liver extracts on production of acid (4 days' fermentation. One unit represents ether extract of 0.1 gram crude liver extract or 0 5 gram corn-steep solids).

were approximately parallel and the titrable acid seemed to be a sufficiently accurate index of growth and fermentation.

Other sources of the growth factor

Wood and Werkman (1935) found that a corn-steep-water medium supported vigorous growth of these organisms, and supplied all their nutritional requirements. Accordingly an

ether extract of steep water was prepared using a concentrated steep which was acidified with H_2SO_4, taken up with $CaSO_4$ and extracted with ether as was done with yeast extract. A dark sticky gum was obtained. The effect of this material is shown in figure 2, *B*. For ease of comparison the activity of all potent materials is expressed in terms of units. These units were chosen so that in the same medium and fermentation time each was approximately equivalent in effect to one unit of yeast factor (ether extract of 1 gram crude yeast extract). One unit of corn-steep extract (containing 18 mgm. of solids) represented the ether extract of 0.5 gram corn-steep solids. Corn steep compared favorably with yeast extract as a source of the growth factor, figure 2, *A*. In ammonium sulfate medium also, its effect was about the same as that of yeast extract. (Data not shown.)

It was observed that liver extract was nearly as good a source of growth factor as yeast extract. Therefore an ether extract of the acidified water-soluble material was prepared and tested for activity. The results in ammonium sulfate and hydrolyzed casein media are shown in figure 2, *B*. One unit represented the ether extract of 0.1 gram liver extract solids, and contained 3.45 mgm. of solids. Distinct stimulation was observed in both media. Koehn and Elvehjem (1936) have fractionated liver extract into hepatoflavine, B_2, and ether-alcohol precipitate fractions, and all of their fractions were tested.[4] The B_2 fraction was the only one able to replace the ether extract of liver extract under the conditions of our experiments. The anti-dermatitis factor (B_2) is not the same as our factor, however, for the former is not ether soluble.

Tatum, Peterson and Fred (1936) found that in a medium containing potato extract and water extract of corn, both extracts were necessary to obtain a good fermentation. The relation of these factors to the yeast stimulant was studied. Ether extracts were made of the acidified concentrated water extracts of potato and corn. Both extracts were active[5] (fig. 3). Growth was more

[4] The authors wish to thank Drs. Elvehjem and Koehn for furnishing these samples.

[5] One unit represented the ether extract (52.8 mgm.) of 100 grams of raw potato and the ether extract (1.58 mgm.) of 1.0 gram of corn.

vigorous with a combination of the extracts than with either alone. Since the combined fractions were more effective than either alone, even though used in twice the concentration of that in the combination, the effect apparently was not due to a single constituent present in both extracts. The difference in the stability of the factors to hot alkali and acid further indicated that two factors were involved. The factor from potato was completely destroyed in normal sodium hydroxide or sulfuric acid when

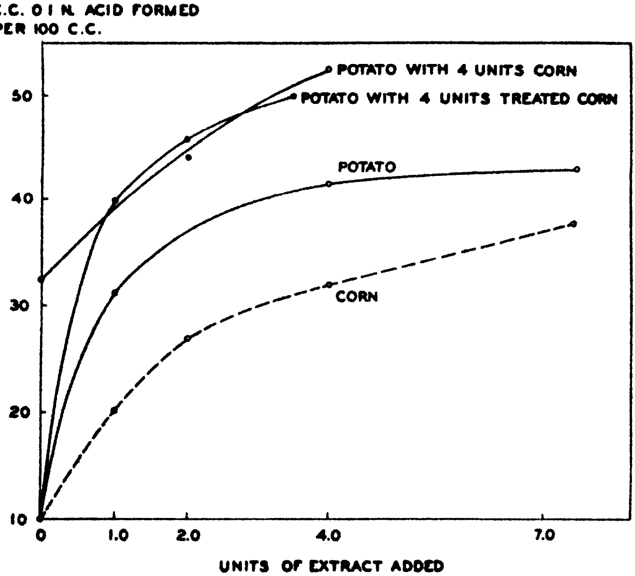

FIG. 3. EFFECT OF CORN AND POTATO EXTRACTS ON PRODUCTION OF ACID IN HYDROLYZED CASEIN MEDIUM

(Four days' fermentation. One unit represents ether extract of 1 gram corn or 100 grams raw potato.)

heated at 136°C. for 45 minutes. The corn extract lost practically all its activity on such treatment, but, when added to untreated ether extract of potato, growth was equivalent to that with the combined untreated potato and corn extracts. Apparently the corn extract contained at least two factors one of which was labile to alkali and acid. The labile factor may be the same as the potato factor. The corn and potato extracts usually do not support growth in the ammonium sulfate medium. In this

respect these extracts differ from those of yeast, corn steep and liver.

The relationship of the ether-soluble corn and potato factors to those present in the Neuberg filtrate of potato and water extract of corn as used by Tatum, Peterson and Fred (1936) with $(NH_4)_2SO_4$ as the only added nitrogen source cannot be determined definitely as yet. On the basis of the original potato used, the Neuberg filtrate was approximately ten times as potent as our ether extract. Two explanations of this difference in activity are proposed. First, the ether extract may have contained only one of several factors present in the Neuberg filtrate. Second, if but one factor is concerned, recovery by ether extraction may have been incomplete. Since hydrolyzed casein was found to be essential for growth in the present investigation it seems probable that amino acids are involved. If they were necessary with Neuberg filtrate of potato as a stimulant, presumably they were present in the water extract of corn.

Relation of growth factor to other biologically active factors

Before further investigation of the properties of the factor it seemed advisable to test other available biologically active substances and to determine their growth-promoting properties under the conditions of our experiments. The following substances[6] have been tested in varying concentrations in the optimal medium with hydrolyzed casein. The limits of concentration used per 100 cc. of medium are given after each compound.

Vitamin B_1	0.05	to	0.7	mgm.
Sporogenes vitamin	0 01	to	2.5	mgm.
Pantothenic acid	0.025	to	1.25	mgm.
Indoleacetic acid	0.01	to	10	mgm.
Inositol	5	to	500	mgm.
Nicotinic acid amide	0.1	to	100	mgm.

None of these substances showed activity under our conditions. The present stimulant is not identical with any one of them, and

[6] The authors wish to thank Drs. A. M. Pappenheimer and R. J. Williams for their contribution of test materials.

probably is not the same as other growth factors which have been reported for microörganisms. The factor for *Staphylococcus aureus* studied by Hughes (1932) and by Knight (1935) is weakly basic as contrasted with the acidic nature of our factor. It does not seem to be related to that described by Koser and Saunders (1935) since veal infusion does not support good growth of propionic acid bacteria.

PROPERTIES OF THE GROWTH FACTOR

The ether extract of yeast extract was treated in various ways, and the effect of such treatments on the activity determined by adding the treated extracts to the basal media. The results are given in table 1.

Stability. The factor was found to be stable to drying on the steam bath but was partially destroyed after 16 hours at 140°C. Similarly, it lost the greater part of its activity when autoclaved in N H_2SO_4 or N NaOH solution. However, the complete activity in ammonium sulfate medium was restored by adding potato extract, which is ineffective by itself in this medium. This indicates that the yeast extract may contain two factors, a labile factor similar to that from potato, and a stabile factor similar to the corn factor. The activity of the potato extract is destroyed by such treatment, while that of corn is not.

Volatility. A water solution of the unneutralized ether extract was distilled with steam. The distillate contained 18.0 cc. 0.1 N acid of the total 56.6 cc. present in the extract. All of the activity was recovered in the residue.

A dried sample of the factor was heated 1 hour at 120°C. under a pressure of 2 mm. mercury in an improvised molecular still. A few drops of a colorless viscous liquid collected on the condenser tube and the residue charred slightly. The residue contained most of the activity, but was partially inactivated by the treatment.

Adsorption and elution. A hot water solution of the factor was treated with Norite (1 gram per 100 cc.). The Norite was washed thoroughly and the filtrates tested for activity. All of the active principle was adsorbed on the Norite. Treatment of the Norite

TABLE 1

Properties of growth factor from yeast

TREATMENT OR FRACTION TESTED*	Medium†	ACID PRODUCED WITH 5 UNITS STIMULANT
		cc. 0.1 N per 100 cc.
Controls:		
Untreated	AS	31
	HC	46
Stability:		
Dried 2 hours on steam bath	AS	31
Dried 16 hours at 140°C	AS	20
Heated 1 hour in 1 N NaOH at 126°C	AS	14
	HC	22
H_2SO_4 treated sample + potato stimulant	AS	32
Volatility:		
Steam distillate	AS	7
Steam residue	AS	30
Vacuum distillate, 2 mm. Hg, 120°C	AS	9
Vacuum residue	AS	21
Adsorption and elution:		
Treated with Norite	HC	9
Eluted from Norite	HC	42
Solubility of free acid:		
Ether extract pH 8.5 (C)	HC	8
Ether extract pH 4.0 (C)	HC	45
Chloroform extract (S)	HC	45
Ether extract (S)	HC	36
Benzene extract (S)	HC	30
Xylene extract (S)	HC	23
Petroleum ether extract (S)	HC	10
Benzene extract (C)	HC	44
Petroleum ether extract (C)	HC	28
Solubility of barium salts:		
85 per cent alcohol extract (S)	HC	46
95 per cent alcohol extract (S)	HC	41
Absolute alcohol extract (S)	HC	19
Acetone extract (S)	HC	17
Absolute alcohol extract (C)	HC	40
Acetone extract (C)	HC	16

* (S) = Single extraction with shaking; (C) = continuous extraction.

† (AS) = Ammonium sulphate medium, 7 days' fermentation. With no stimulant 7.0 cc. 0.1 N acid produced.

(HC) = Hydrolyzed casein medium, 4 days' fermentation. With no stimulant 10 cc. 0.1 N acid produced.

with hot 50 per cent alcohol containing a little acetic acid eluted the factor, which was completely recovered. Koser and Saunders (1935) used a similar procedure for purification of the factor from veal infusion. We were unable to purify, our factor markedly in this way. A considerable portion of the original material was adsorbed and eluted, including the succinic acid.

A water solution of the factor was passed through a 20 cm. Norite column and washed with water. The column was divided into three segments, each segment eluted as before, and the activity of each fraction tested. The activity was distributed throughout the column, so that such a method did not seem to have any promise for purification of the factor.

Acidic nature. In order to prove that the factor was acidic, water solutions adjusted to pH 8.5 and 4.0 were extracted continuously with ether. The extraction was complete at pH 4.0, but the extract at pH 8.5 was inactive. The corn, potato, and liver fractions all behave similarly, indicating that all factors are acidic.

Solubility of free acid. Dried samples of the factor (3 units) were shaken with 50 cc. of various solvents and allowed to stand for 24 hours. Water solutions of these extracts were tested for activity. The results in table 1 show that solubility in chloroform, ether, benzene and xylene decreased in the order given. The factor is not soluble in petroleum ether under these conditions. Continuous extraction with benzene of a sample dried on filter paper was complete in 4 hours. A similar continuous extraction with petroleum ether for 24 hours failed to remove the active principle completely. Benzene seemed to be the most selective of these solvents, and may be of value in further purification.

Solubility of barium salts. Water solutions of 3 units of the factor were neutralized to pH 7.3 with barium hydroxide, dried, and extracted with 50 cc. of various concentrations of alcohol and with acetone. The results show that the barium salt of the factor was soluble in 85 or 95 per cent alcohol, but not very soluble in absolute alcohol or acetone. Recovery with acetone was incomplete even with continuous extraction for 24 hours.

PRELIMINARY PURIFICATION OF THE YEAST FACTOR

An intensive attempt has not been made to purify the factor but several methods suggested by its properties have been tried. Table 2 lists the extent of the purification brought about by some of these procedures. It should be emphasized that the weights of the fractions given in this table are probably of no particular value except in indicating the quantities of impurities removed. The activity of the stimulant is probably very much greater than these weights would indicate.

TABLE 2

Preliminary purification of yeast factor

TREATMENT	FRACTION TESTED	WEIGHT OF ONE UNIT*	ACIDITY OF ONE UNIT	ACID PRODUCED WITH 5 UNITS OF FACTOR†
		mgm	*cc. 0.1 N*	*cc. 0.1 N per 100 cc. medium*
None		63 0	7 5	46
Calcium salts precipitated with 85 per cent alcohol	Filtrate	22 7		47
Barium salts precipitated with 85 per cent alcohol	Filtrate	14.7	0 92	45
Barium salts precipitated from alcohol with acetone	Precipitate I	14 3		44
	Precipitate II			25
	Filtrate I	1 5		32
	Filtrate II	8 5	0 75	36
Free acid extracted with benzene	Extract	6.3	0 36	29
Benzene extract precipitated from ether with petroleum ether	Precipitate		0 23	28
	Filtrate		0 13	10

* One unit represents ether extract of 1 gram yeast extract.

† Hydrolyzed casein medium; with no stimulant 10 cc. 0.1 N acid was produced.

Considerable purification was possible by precipitating solutions of the calcium or barium salts from 85 per cent alcohol. The active factor was recovered from the filtrate of the calcium salts by acidification and extraction with ether, and from the barium salts filtrate by removing the barium with sulfuric acid. Both of these treatments removed succinic acid and other impurities and gave an appreciable purification with complete recovery of the activity.

The insolubility of the barium salts of the factor in acetone suggested precipitating the barium salts from an 85 per cent alcohol filtrate with acetone. An alcohol solution containing 5 units of stimulant as the barium salts was treated with 14 volumes of acetone and precipitate I and filtrate I, table 2, were separated. Precipitate II and filtrate II were prepared by treating an alcohol solution containing 300 units with 3 volumes of acetone. The active factor was recovered from all fractions by acidification with sulfuric acid and extraction with ether. All fractions were active, but precipitation with 14 volumes of acetone was most complete. Filtrate I was the most active on the basis of its dry weight. It is not known whether the factor was fractionated by this precipitation or whether precipitation was merely incomplete.

The insolubility of the factor in petroleum ether suggested precipitating it with this solvent. Two units of benzene extract (S, table 1) were evaporated to dryness, taken up in ether, concentrated to 5 cc. and 10 volumes of petroleum ether were added. The oily precipitate which separated on the sides of the flask contained all the active material of the benzene extract.

Response of other propionics to the growth factor and the necessity of amino acids for growth

In a previous paper (Tatum, Wood and Peterson (1936a)) it was shown that the potato stimulant was effective for all strains of propionic acid bacteria which were tested. This is also true of the ether-soluble yeast factor. The following cultures described by Hitchner (1934) have been tested.

	cc. 1 0 N acid per 100 cc.
No. 11. *Propionibacterium pentosaceum*	20
No. 33. *Propionibacterium freudenreichii*	21
No. 52. *Propionibacterium thönii*	12
No. 54. *Propionibacterium jensenii*	14
No. 56. *Propionibacterium zeae*	24
No. 57. *Propionibacterium pentosaceum*	22
No. 61. *Propionibacterium arabinosum*	26

The cc. of 0.1 N acid produced in $(NH_4)_2SO_4$ medium in 4 days with 3 units of stimulant are given after each culture. The

bacteria used for inoculation of these tubes have been transferred twice on the $(NH_4)_2SO_4$ medium. This prevented carrying over the yeast extract with the inoculum. By this method growth in this medium without the addition of the factor was completely eliminated and the titration was 1.0 cc. These results show that the ether-soluble yeast factor stimulates growth of these cultures and indicate that the stimulant may be effective for all strains of propionic acid bacteria. The factor is apparently essential for growth on $(NH_4)_2SO_4$ medium. Some additional factor is involved, however, for the cultures did not grow indefinitely in this medium. All failed to grow after the third transfer. If hydrolyzed casein were added to the medium all the cultures grew vigorously and there was no decrease in activity during repeated transfers. This would seem to indicate that amino acids are essential and that there was a carry over from the yeast extract with the inoculum which was sufficient to allow growth on the first and second transfers. The ether extract of yeast extract is free of amino acids as shown by the ninhydrin reaction, but it does contain some nitrogen. Further explanation of these results will be offered in a subsequent paper (Tatum, Wood and Peterson (1936b)).

Application of results to fermentation studies

Studies of fermentation mechanism have often led to confusing results because of the complex media used for the growth of the propionic acid bacteria. In many cases so large an amount of unknown material was present in the medium that it was often impossible to decide whether certain products were derived from the sugar or from the unknown material. For example, van Niel (1928) concluded that the succinic acid produced by propionic acid bacteria originates from the yeast extract in the medium, while other investigators have claimed that it is of carbohydrate origin. The donation or acceptance of hydrogen by unidentified compounds of the medium also has confused interpretation of fermentation data and made the calculation of oxidation-reduction balances of doubtful significance. If a fairly active fermentation of glucose were possible with only a small quantity of un-

known material in the medium these problems could be more easily investigated and the results would be of more significance.

The data in table 3 show that some propionic acid bacteria will ferment glucose readily in a simplified medium containing only the ether-soluble yeast factor as a source of unknown carbon. It seems probable that cultures 11, 33, 56, 57 and 61 would ferment two per cent glucose completely in this medium with a somewhat longer period of incubation. All fermentations were more vigorous in the presence of hydrolyzed casein.

With the most active preparation of the growth factor thus far obtained (85 per cent alcohol soluble barium salts, table 2),

TABLE 3

Fermentation of glucose in simplified media by various propionic acid bacteria

MEDIUM*	GLUCOSE FERMENTED PER 100 CC.							
	Culture 9	Culture 11	Culture 33	Culture 52	Culture 54	Culture 56	Culture 57	Culture 61
	grams	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*
Ammonium sulphate medium ..	0 25	1 81	3 18	0 37	0 45	1 57	1.49	1.34
Hydrolyzed casein medium. ..	1.85	2 85	3 34	1 30	1 24	2 80	3 02	2.70

* Ammonium sulphate medium—3 units of growth factor per 100 cc.; 3.5 per cent glucose, 0.3 per cent $(NH_4)_2SO_4$, mineral salts, excess $CaCO_3$, 10 days' fermentation.

Hydrolyzed casein medium; composition same as ammonium sulphate medium with 0.9 per cent hydrolyzed casein added.

only 44 mgm. of unknown material would be present per 100 cc. of medium. Further purification of the factor will probably reduce very materially the quantity of unknown material required.

SUMMARY AND CONCLUSIONS

An ether-soluble factor has been obtained from yeast extract which is indispensable for growth of all tested cultures of propionic acid bacteria on a synthetic ammonium sulphate medium. The addition of hydrolyzed casein greatly improves growth and makes repeated transfer possible in the supplemented synthetic medium.

The factor has been found in all materials investigated (yeast extract, corn steep, potato extract, corn extract, and liver extract) which support good growth of propionic acid bacteria.

The growth factor is a non-volatile, ether-extractible acid. The free acid is soluble in chloroform, ether, benzene and xylene, but almost insoluble in petroleum ether. The barium salt is soluble in 95 per cent alcohol but sparingly so in acetone. The factor is adsorbed on Norite and may be eluted with acid-alcohol.

The factor can not be replaced by hepatoflavine, vitamin B_1, sporogenes vitamin, pantothenic acid, indoleacetic acid, inositol or nicotinic acid amide.

Since vigorous growth and fermentation can be obtained in a synthetic medium with only small amounts of unknown material, such a medium should be of value in quantitative studies on the mechanism of the propionic acid fermentation.

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Edwin Oakes Jordan
1866–1936

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1866–1936

The death of Edwin Oakes Jordan on the second of September, 1936, removed a distinguished member of the second generation of American bacteriologists. Leader, scholar and teacher, he early took a prominent position in the development of bacteriology and left it materially advanced by his fundamental contributions.

Dr. Jordan was born in Thomaston, Maine, on July 28, 1866. His training in science led to the bachelor's degree at the Massachusetts Institute of Technology, conferred in 1888. Here he came under the tutelage of William T. Sedgwick, pioneer in the field of public health, whose influence remained with him throughout life. He was associated with Sedgwick on the staff of the Massachusetts State Board of Health in investigations on water purification and sewage disposal, and worked from 1888 to 1890 at the experimental plant at Lawrence, the first of its kind in the country. Early interest in the broad aspects of biology was evidenced by his taking up studies in zoology under Charles O. Whitman at Clark University, where he was a fellow until the attainment of the degree of Doctor of Philosophy in 1892.

William Rainey Harper was at that time gathering his faculty for the establishment of the University of Chicago and Dr. Jordan accepted the appointment of associate in the new Department of Anatomy. He delighted in later years in telling of the competition among the new instructors for the use of the kitchen sinks available for laboratory purposes in the apartment house first occupied by the science departments. Science buildings were soon available on the campus, and the teaching and investigations in bacteriology were moved about from attic to basement

[1] President of the Society of American Bacteriologists, 1905.

until the establishment of the Department of Hygiene and Bacteriology in Ricketts Laboratory in 1915. Meanwhile, Dr. Jordan was appointed to the rank of Assistant Professor in bacteriology in 1895, Associate Professor in 1900, and Professor in 1907, assuming the Chairmanship of the newly formed department upon its organization in 1914. This position he retained through the succeeding years until retirement in 1933 as Professor Emeritus. The growth of this department represented his personal leadership and scientific attainment. Its progress is illustrative of the development and recognition of the science of bacteriology in American universities, in which he took an influential part on a national scale.

Dr. Jordan's breadth of vision explains the varied interests displayed in his investigations. Not only did he deal with the details of bacterial study, but he also had the ability to envisage the significance of bacteria in the broad field of public health. These interests ran concurrently and we find publications on the study of intestinal bacteria along with articles on water purification; on the metabolism of the Pfeiffer's bacillus while work was appearing on epidemic influenza; and on the antigenicity and classification of the paratyphoid bacilli throughout extended work on the broader aspects of food poisoning. Articles came from his pen on water purification, sewage disposal, pasteurization of milk and the bacteriology of typhoid fever. Instrumental in the early establishment of the basic principles of sanitation, he maintained this position of leadership throughout life and was consulted on problems presented by the application of these principles.

In later years, Dr. Jordan turned to the consideration of respiratory diseases and led an extensive series of studies on the bacteriology of the upper respiratory tract and on the bacteriology and epidemiology of influenza and the common cold. His book "Epidemic Influenza" stands as a classical summary of our knowledge of that subject to the time of its publication in 1927. Statistical analyses of the prevalence of diphtheria and typhoid gave him an opportunity to consider the ebb and

flow of these diseases under the influence of modern measures of control.

From his monumental investigations in the self-purification of streams to the analysis of bacterial variation and oxidation-reduction potentials Dr., Jordan was in the van of bacteriologic thinking, and to the last his perspective was not altered nor his activity diminished. It was not often in recent years that he put his hand to laboratory work, but through the reliability of well-trained assistants and his own laboratory sense, he effectively conducted investigations and gave expression to his experimental interest. His writings disclose not only an ability to analyse in the laboratory, but also to synthesize in community applications. The soundness of his contributions was based on his wealth of knowledge of the literature; he read widely in several languages and built an extensive library in bacteriology and public health.

Many of Dr. Jordan's studies were carried on with associates and students. In the solution of the problems with which he had an intimate relation, he took a leading part and gave the work his stamp of authority. More than a half-hundred graduate students received their Doctor of Philosophy degree under him, from 1900 to his retirement. More often than not, he encouraged the student to work out his own program of training and problem of investigation. As a result, the student usually developed his own individuality, but Dr. Jordan by direction and precept taught him the basic principles of scientific thought, procedure and expression. Thus, his students were not cast in the same mold and have since scattered to a variety of activities; but they all bear the mark of a scientific training.

Dr. Jordan did not carry a heavy burden of class teaching but while not a fluent speaker, his use of beautiful English, his insistence on the teaching of principles and the logical arrangement of content, made his lectures models of the formal type of teaching. Writing was the form of expression in which Dr. Jordan excelled. Among contemporary American scientists capable of using the English language at its best, he took a

high rank. He was a purist in his choice of words and forms of expression; precision, directness, and clarity of thought were attributes of his writing; and his scientific articles were examples of logical analysis of data and conservative deduction.

The textbook, "General Bacteriology," which first appeared in 1908 and was finally presented in 1935 in its eleventh edition, has been widely accepted as a standard, and is universally recognised for its clear and readable style. A further contribution was his masterful book on "Food Poisoning and Food-Borne Infection," first published in 1917 and revised in 1931. It stands as a signal contribution by the leading authority on food poisoning in this country, a field to which he added so much by his personal investigations. In 1928, Dr. Jordan as senior editor brought together in one volume, entitled "The Newer Knowledge of Bacteriology and Immunology," the summarized work and opinions of eighty-two contributors, specialists in their respective fields. This detailed compilation was immediately accepted as an authoritative and highly valued book of reference. He was joint editor of the Journal of Infectious Diseases from its inception in 1904, and editor of the Journal of Preventive Medicine from 1926 to 1933. When the latter journal was combined with the American Journal of Hygiene, he assumed the editorship of its section on epidemiology.

The leadership of Dr. Jordan was exerted not only through his teaching, writing and editing, but also through connections with scientific organizations. He was one of the founders of the Society of American Bacteriologists and its president in 1905. He was also concerned in the founding of the Epidemiological Society, in which he took great interest and of which he was president in 1930. A member of the American Public Health Association since 1899, Dr. Jordan was active in the Laboratory and Epidemiology Sections. For years he served on the Association's committee studying water analysis and joined in the publication of the frequently revised reports of this committee on "Standard Methods of Water Analysis." He gave of his counsel to the Medical Fellowship Board of the National Research Council. He was an active member of the International

Health Board of the Rockefeller Foundation from 1920 to 1927 and upon the organization of the International Health Division, he was appointed to its Board of Scientific Directors and served from 1930 to 1933. He was a loyal collaborator in various activities of the American Medical Association and was on the Council on Foods from 1933 to the time of his death. Space does not permit the enumeration of other national and international groups which sought Dr. Jordan's advice and listed his name among their leaders. The medical history of Chicago was also enriched by Dr. Jordan's contributions to its organizations. He served as trustee of the John McCormick Institute for Infectious Diseases. In 1906, he was elected President of the Chicago Pathological Society and in 1932 held the same office in the Institute of Medicine of Chicago. He was retained by the Sanitary District of Chicago at the turn of the century during the controversy over the pollution of the Illinois River by the Chicago drainage canal; it was in this connection that he and his associates made fundamental observations on the self-purification of streams. The Chicago Board of Health frequently sought his advice and in his later years he was a member of the Board.

In all these contacts, whether with scientific, philanthropic or civic organizations, Dr. Jordan gave generously from his great fund of practical knowledge, his wide acquaintance with men and their writings, and his exceptional judgment. His advice, much sought, was given quietly and with the force of logic. An analytic mind, an unusual memory and an ever-present tact combined to make his counsel valuable and his opinion respected.

Numerous honors were conferred on Dr. Jordan, in recognition of his activities and scientific achievements. Before his retirement from the faculty of the University of Chicago, he held the Andrew MacLeish Distinguished Service Professorship. In 1934, the American Public Health Association bestowed on him the Sedgwick Memorial Medal, awarded for distinguished service in public health. The honorary degree of Doctor of Science was conferred on him in 1920 by the University

of Cincinnati. He was more recently elected to honorary membership in the Society of American Bacteriologists. In the spring of 1936, the National Academy of Sciences extended the last of his honors by electing him to membership.

Dr. Jordan was not a man to seek such honors. Deeply concerned in his own scientific endeavors, he gained pleasure in accomplishment. Modest, courteous and almost timid, he was considered by strangers as distant and formal. Acquaintance led to the discovery of a most genial personality, delightful in conversation, alert in repartee, and resourceful in humor. His knowledge of the classics and contemporary literature, his wide acquaintance with the sciences, his interest in the various expressions of art, and his love of travel and sports combined to furnish him with a variety of information on which he called with facility. To his students he was kindly and earnest, and by them he was admired and respectfully known as "The Chief." By his close associates and friends, he was held in affection as a charming personality. Many were the expressions of homage made by his students, colleagues and other friends who joined in securing Dr. Jordan's portrait in 1934 for presentation to the University of Chicago.

Dr. Jordan is survived by his wife, Elsie Fay Pratt Jordan, whom he married in 1893, and by three children, Henry Donaldson, Edwin Pratt and Lucia Elizabeth Dunham.

With the death of Dr. Jordan, bacteriology has lost a scientist, mankind a humanitarian, literature a scholar, and his associates a deeply-valued friend.

N. Paul Hudson

CULTIVATION OF CELLULOSE-SPLITTING BACTERIA ON MEMBRANES OF ACETOBACTER XYLINUM

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Received for publication August 10, 1936

Cellulose-splitting bacteria are present in fairly large numbers in every cultivated soil. It is easy to demonstrate their presence by means of crude cultures but to isolate them in pure cultures is one of the very difficult technical problems in bacteriology. In spite of the great interest of soil-bacteriologists in this group of bacteria, a pure culture was not attained, according to Fehér, until 1919 (by Hutchinson and Clayton) and two additional microörganisms of this group have been isolated (by Winogradsky) only in 1929. Even today, owing to the unsatisfactory culture methods, the purity and genuineness of some of the strains described are still questioned.

The culture media which have been used till now for the isolation of cellulose-splitters consisted of silica jelly or purified agar-agar as a base with the addition of an inorganic salt solution and pure cellulose as the only source of carbon. Cellulose was added in the form of teased filterpaper or as a powder precipitated from a solution of cellulose in H_2SO_4 or Schweizer's reagent. The presence of cellulose in either form causes a turbidity in the medium which makes it impossible to detect the early stages of colony development. Moreover, since cellulose, even in the precipitated form, is a fairly compact material, it takes at least 8 days before the first signs of decomposition of the cellulose can be observed. A long time is, therefore, required to identify and pick out the cellulose-attacking colonies, and in the meantime they may be overgrown by associated bacteria, which are invariably present in the crude cultures and which multiply quicker than the cellulolytic bacteria. Hence the difficulty in obtaining pure cultures.

In this paper a new culture medium is described, based on the use of a new kind of cellulose which permits an absolutely certain identification of cellulose-splitting bacteria in 48 hours. Further advantages of this medium are its transparency and reflecting surface, which make it possible to detect very young colonies and to test their purity *in situ*. The medium is prepared from the jelly-like membranes produced by the *xylinum* group of the acetic acid bacteria cultivated in fluids containing alcohol or sugar. These membranes are known to consist mainly of a cellulose which has very peculiar properties. An undried membrane, 9 cm. diameter and 3 mm. thickness, has a weight of *ca.* 18 grams. After drying, the same membrane is a delicate film of paper only 0.03 mm. thick, weighing 0.13 gram. A fresh membrane may, therefore be said to be a film of paper the thickness of which has been increased 100 times by water of imbibition. Every slight effect of decomposition, which on the dry cellulose is practically invisible, is seen on the wet membrane in the same magnification, that is to say, increased 100 times and is thus made conspicuous. For this reason a medium prepared from these membranes already shows decomposition of cellulose 48 hours after inoculation at places where colonies of cellulose-splitting bacteria develop. At these points the cellulose becomes liquefied in a manner similar to the liquefaction produced by colonies of proteolytic bacteria on a gelatin plate.

Colonies transferred from the liquefied areas on the membrane to a medium containing filter paper as the only source of carbon grew readily and in due time caused decomposition of the filter-paper.

TECHNIQUE FOR PRODUCING THE MEMBRANES

A nutrient solution was prepared consisting of: 100 cc. tap-water; 0.5 gram "Marmite" or any other yeast extract; 0.25 cc. acetic acid and 3 to 5 grams cane sugar. After sterilization in the autoclave 2 per cent alcohol was added and the medium distributed to a height of 5 cm. in sterile wide-mouth cotton-stoppered bottles, which should preferably have the same inner diameter as a Petri dish. The fluid was inoculated with *Acetobacter xylinum* and cultivated at 30°C.

Under these conditions growth of *A. xylinum* takes place in the form of a tough, gelatinous membrane covering evenly the entire surface of the fluid and increasing enormously in volume as growth proceeds. This membrane is removed with a sterile spatula or forceps as soon as it reaches a thickness of about 2 mm. Without reinoculation new membranes are repeatedly formed so long as sugar or alcohol are present in the solution. If these materials have become exhausted, a part of the fluid is poured off and fresh medium added to the original level. As only a few microörganisms are able to develop in this acid medium, air contaminations are rare and the same bottle can be used for a long time and a large number of membranes obtained.

The strain of *A. xylinum* used in these experiments was isolated from local vinegar. It showed a very rapid growth, the time required for the formation of a membrane of about 2 mm. thickness being not more than 2 to 3 days. For thicker membranes a correspondingly longer time was required.

The membranes removed from the culture bottles were treated, while still wet, in the following manner: They were washed for 1 hour in running tapwater and then immersed for 1 to 2 days in 5 per cent NaOH. The alkali dissolves the bacterial proteins without attacking the thick wall of cellulose which covers the individual bacteria. From the NaOH solution the membranes are transferred, after thorough rinsing in tapwater, into dilute HCl in order to remove traces of the alkali. The membranes remain in the acid water for about 2 hours after which the acid is washed out completely in running tapwater; the progress of the final washing is followed by adding phenol red or simply strips of litmus paper to the washwater. After purification the membranes are put into wide mouth bottles containing the following salt solution: 1 gm. NH_4Cl; 1 gram K_2HPO_4; 0.5 gram $MgSO_4$ in 1 liter of water. In this solution the membranes are sterilized in the autoclave and kept until needed.

For use as culture plates the membranes are taken from the bottle and, after the excess of fluid has been drained off, are spread on the bottom of a Petri dish and again sterilized in the autoclave. These plates are then treated as ordinary agar plates. They are dried in the incubator upside down until the surface is

freed of moisture and inoculated with an ordinary platinum loop or a bent glass rod.

For determining the number of cellulose-splitting organisms in any fluid, 0.2 cc. of the fluid or of a dilution is dropped on the plate. In contrast with an agar plate the fluid is quickly absorbed and the surface of the medium remains dry, so that even highly motile bacteria remain fixed. Wherever colonies of cellulose-splitting bacteria develop, liquefaction of the cellulose becomes visible and the number of cellulose-splitting organisms in the fluid examined can be calculated from the number of such points of liquefaction.

SUMMARY

A new solid culture medium prepared from "*xylinum*" cellulose is described which permits easy and rapid identification of cellulose-splitting organisms. The identification is based on the ability of such organisms to liquefy the medium, which effect can be observed as early as 48 hours after inoculation. The new medium is equally useful for the isolation of pure strains and for the determination of the number of cellulose-splitting bacteria in any substance.

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THE BACTERIOSTATIC AND BACTERICIDAL ACTION OF GREAT SALT LAKE WATER

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Received for publication August 31, 1936

Great Salt Lake in northern Utah is an extensive and extremely saline body of water having considerable geological, historical, commercial and biological interest. The water usually contains more than 20 per cent salt and in December 1935 when the water for the experiments reported below was collected, the salinity was 27.6 per cent. In reviewing the literature of bacterial life in strong brines Hof (1935) shows that most species which have been tested tolerate no more than 6 per cent salt and he concludes that 15 per cent salt limits the activities of halophiles with very few exceptions. Previous to the work of Daines (1917) who recovered as many as 625 bacteria per milliliter of water from Great Salt Lake, it was believed to be sterile. However, inasmuch as he isolated these bacteria on 2.5 per cent NaCl nutrient agar, his investigations failed to show whether the bacteria were halophiles indigenous to the lake or if they were merely halotolerant contaminants. In a survey covering a large number of samples collected during the last year from different depths and stations, Smith (1936) almost invariably found a few hundred bacteria per milliliter of lake water which developed on lake water media.

Using a direct microscopic technique Smith and ZoBell (1936) have demonstrated the occurrence of autochthonous bacteria in Great Salt Lake which are capable of multiplying to form microcolonies on glass slides submerged in the brine. In continuing these studies it is desirable to know if the bacteria found in the

lake are obligate halophiles which are foreign to other habitats or if the water also contains appreciable numbers of halotolerant transplants from soil, sewage or other less saline environments. The problem also has a public health aspect since the unusual properties of the brine annually attract thousands of bathers to the resorts. Considering this aspect, Frederick (1924) records that the etiological agents of certain ocular, respiratory or gastro-intestinal infections remain viable in lake water for extended periods and some of them actually multiply in lake water. However, she used massive inocula of stock cultures and other questionable techniques which may invalidate her conclusion that there is a real danger of infection from undiluted lake water.

EXPERIMENTAL

In order to determine to what extent bacteria from different habitats multiply in Great Salt Lake water, nutrient media were prepared having the following composition:

Bacto-peptone	3.0 grams
Proteose-peptone	2.0 grams
Beef extract	2.0 grams
Bacto-agar	5-12.0 grams
Water	1000.0 ml.

Either lake water, diluted lake water, distilled water or sea water from the Pacific Ocean was used. The reaction of all the media was adjusted to pH 7.5. Lake water must be heated higher than 100° in an autoclave to dissolve the agar. At first considerable difficulty was experienced with the lake-water nutrient agar which congealed prematurely. Undiluted lake water containing 10 grams of Bacto-agar per liter congeals within a few minutes at 60° and even in lake water diluted one-half with distilled water, 10 grams of agar gives a medium which congeals before it can be cooled to 45° for plating. Five grams of agar per liter were found to be adequate to solidify lake water medium and it is necessary to cool it rapidly to 45° and pour it immediately or it will congeal in the tube. Six grams of agar per liter were adequate to solidify the 75 per cent lake water medium (750 ml. lake water plus 250 ml. distilled water); 7.5 grams were used in

the 50 per cent lake water, and 10 grams per liter in more dilute lake water.

The different media were used to plate identical samples of raw lake water, sewage, sea water, soil and the pooled washings from the mouths of two individuals. Plates inoculated with the oral flora were incubated at 37° and the others at 25° until the counts became fairly constant. In general, the sewage bacteria developed most rapidly, there being little increase in the colony counts after four days. The bacteria from the lake developed most slowly, requiring two to three weeks for the colony counts to approach constancy; and even after prolonged incubation the majority of the lake water bacteria formed only minute colonies.

TABLE 1

Number of bacteria per milliliter of Great Salt Lake water which developed on nutrient agar prepared with different concentrations of lake water (L.W.), distilled water or sea water

SAMPLE NUMBER	75 PER CENT L.W.	50 PER CENT L.W.	25 PER CENT L.W.	10 PER CENT L.W.	DISTILLED WATER	SEA WATER
183	162	154	95	32	10	8
185	24	20	16	4	1	1
186	33	38	29	2	0	2
187	266	247	109	16	3	5
197	224	214	125	17	11	7
198	290	285	146	41	8	14

The plate counts on six samples of lake water on the different media are given in table 1. The water was collected in glass bottles from the end of the Salt Company pier near Saltair resort in December 1935 at which time the water temperature was 1.2°C. Although bacterial colonies appeared on the undiluted lake water medium, their small size and the crystallization of salt made it impossible to enumerate them. The highest counts were obtained on the 75 per cent lake water medium. Almost as many lake bacteria developed on the 50 per cent lake water medium, or that which was diluted one-half with distilled water. With further dilution the counts decreased sharply. There was an average of 3.8 bacteria from the lake water samples

which grew on distilled water medium for each 100 which grew on the 75 per cent lake water medium. Unlike the typical lake bacteria, most of the colonies which developed on the distilled water medium appeared within two or three days and were quite large. As a matter of fact, the designation "distilled water medium" in this experiment is misleading because when 15 ml. of nutrient medium prepared with distilled water is inoculated with 1.0 ml. of raw lake water having a salinity of 27.6 per cent, the resulting medium actually contains about 2 per cent salt. When some of the colonies which developed on the so-called distilled water medium were emulsified in sterile water and streaked on 75 per cent lake water medium, only a small percentage of

TABLE 2

Relative numbers of bacteria from different sources which developed on nutrient agar prepared with various dilutions of Great Salt Lake water (L.W.), distilled water and sea water, expressed as ratios to the plate counts on the best medium expressed as 100

SOURCE OF SAMPLE	75 PER CENT L.W.	50 PER CENT L.W.	25 PER CENT L.W.	10 PER CENT L.W.	DISTILLED WATER	SEA WATER
Sewage	0.0	0.0	6 7	18 1	**100.0**	9 5
Soil	0.0	0.8	12 6	29.8	**100.0**	13 7
Oral cavity	0.0	0.0	3.9	15.6	**100.0**	7 6
Pacific Ocean	0.2	0.6	10.4	64.3	6.2	**100.0**
Great Salt Lake	**100.0**	96.4	51.2	11 3	3 8	4.1

them grew although all of them grew when transplanted on freshwater medium. This is believed to indicate that at least some of the bacteria from the lake which develop on distilled water media are halotolerant freshwater varieties.

Table 2 summarizes the relative numbers of bacteria from sewage, soil, oral cavity, the Pacific Ocean and Great Salt Lake which developed on the different media. The averages of two or more determinations are given expressed as ratios on a basis of 100 representing the number of colonies on the respective medium which proved to be best. The highest counts of sewage, soil and mouth bacteria were obtained on the medium prepared with distilled water. Therefore the microflora from these sources

will be referred to as freshwater microflora. Virtually none of the freshwater bacteria grew on the more concentrated lake water media. Even 25 per cent lake water inhibited most of them. The number of bacteria from soil, sewage or the oral cavity which were capable of growing in 50 to 75 per cent lake water media was too small and variable to permit expression although occasionally such halophilic varieties are encountered. Soil bacteria were more euryhaline than those from the oral cavity or sewage. Only 0.23 per cent of the bacteria found in 11 different swimming pools in Salt Lake City and 1.84 per cent of those in the unchlorinated municipal water supply grew on 50 per cent lake water medium.

It is of interest to note that the sea water medium was more bacteriostatic than the 10 per cent lake water medium for all the microflora except those from the sea although both kinds of media had approximately the same salinity and osmotic pressure. It has been shown by Lipman (1926), Korinek (1927) and others that the bacteria in the sea differ from freshwater varieties in their ability to live in sea water. According to ZoBell and Feltham (1933) who made similar observations on the specificity of marine bacteria, sea water possesses some factor besides its salt concentration which inhibits non-marine bacteria and which favors marine species. While most of the bacteria in the lake are obligate halophiles as indicated by the fact that few of them developed on media hypotonic to lake water, they do not require lake water media for their multiplication because many of them developed on isotonic sodium chloride media.

When lake water lactose broth was inoculated with 1.0 ml. quantities of raw sewage there was rarely any evidence of fermentation and never 10 per cent gas production although freshwater lactose broth revealed the presence of thousands of gasifying *Escherichia coli* per milliliter of the sewage. Attempts to recover *E. coli* from the inoculated lake-water lactose broth after 48 hours by streaking on standard E.M.B. plates were all negative. This indicates that lake water is bactericidal as well as bacteriostatic for *E. coli*. Similarly pure cultures of recently isolated *E. coli* and *Staphylococcus albus* were inoculated into

lake water nutrient broth of different concentrations. In each case a loopful of the organisms from young agar slants was suspended in 10 ml. of sterile saline and then a loopful of the resulting suspension was used to inoculate tubes of lake water broth, care being taken not to touch the inside walls of the tube with the inoculating loop. These were incubated at 25°C. since all of the stock cultures were able to grow at this temperature and since this approximates the temperature of the lake water in the summer time. After four days there was no evidence of growth in the 25 per cent or higher concentrations of lake water broth. *S. albus* clouded the 10 per cent lake water broth a little. Streaking loopfuls of the broth on nutrient agar revealed that the 50 per cent as well as the undiluted lake water broth was sterile but viable *S. albus* and *E. coli* were recovered from the 25 per cent lake water broth. The number of organisms recovered was only a small fraction of the number originally present, showing that even in the presence of peptone, 25 per cent lake water is bactericidal. Old stock cultures of *E. coli* were found to be much more salt tolerant than the recently isolated strains and even the latter were more resistant than those occurring naturally in sewage.

Field observations reported by Smith (1936) also indicate that sewage bacteria cannot tolerate lake water. He inoculated 23 samples of lake water collected near places of sewage pollution into standard lactose broth none of which was fermented. Likewise all were negative for *E. coli* when streaked on E.M.B. plates. A search of the Utah State Board of Health laboratory records reveals that *E. coli* has not been found in undiluted lake water. However, it should be recognized that under certain conditions inflowing freshwater may actually flow over the surface of the denser lake water for considerable distances thereby forestalling the mixture of the salt water with the freshwater bacteria. Also the bactericidal potency of the lake water in nature will be greatly reduced by high concentrations of organic matter and by dilution with more than three volumes of freshwater.

Quantitative data on the bactericidal effect of lake water were obtained by seeding it with appropriate dilutions of sewage or other sources of freshwater bacteria. After various periods of

exposure, 1.0 ml. quantities were plated out on standard fresh-water nutrient agar. Controls were run consisting of "formula C," a balanced salt solution which Butterfield (1932) has found to be superior to other dilution waters for the prolonged survival of water bacteria. The plates were incubated for four days at 25° and the colon es counted. Table 3 summarizes typical findings on the death rate of sewage bacteria in different concentrations of lake water. The results are expressed as ratios on a basis of the number of bacteria surviving in the "formula C" control being 100.

TABLE 3

Relative numbers of sewage bacteria which survived exposure to Great Salt Lake water (L.W.) of different concentrations for various periods of time expressed as ratios to the number which survived in "formula C" expressed as 100

TIME OF EXPOSURE	"FORMULA C"	LAKE WATER	75 PER CENT L.W.	50 PER CENT L.W.	25 PER CENT L.W.	10 PER CENT L.W.	5 PER CENT L.W.	DISTILLED WATER
1 minute	100.0	4.8	7.6	19.9	60.0	64.8	85.1	99.2
15 minutes	98.2	2 3	6 1	12 5	38.7	58.7	63.6	93.9
30 minutes	97.4	1.2	6.0	13.0	33.0	54.2	63.2	72.3
1 hour	96 3	1.0	2 9	10 2	27.2	46.5	71.4	74.8
2 hours	99.5	1.1	1.9	8.9	20.6	41.1	76.5	60.7
6 hours	103 8	0 4	0.7	4.2	13 7	35.1	80.0	83.9
24 hours	117.6	0.0	0.0	0.8	10.4	23.8	87.1	93.7

Great Salt Lake water destroyed the viability of the majority of the sewage bacteria almost immediately. One minute's exposure to undiluted lake water killed over 95 per cent of the bacteria. It is recognized that part of the apparent bactericidal effect may be due to the bacteriostatic action of the lake water which is carried over with the inoculum, because when 15 ml. of nutrient agar is inoculated with 1.0 ml. of lake water, the medium contains about six per cent lake water or two per cent salt, and this is known to inhibit the growth of some sewage bacteria. However, bacteria do not multiply after being exposed to more dilute lake water which would not carry enough salt into the medium to inhibit growth. Therefore most of the decrease in the counts can be attributed directly to the bactericidal effect of

lake water. The continued decrease in the counts with increasing time of exposure lends support to this contention.

Tests were also made on the viability of other freshwater microflora in lake water. Table 4 shows that the mixed microflora from the oral cavity dies off rapidly in lake water although a few varieties survive six hours' exposure. In general, there was a larger proportion of halotolerant bacteria in the mouth than in sewage. About 60 per cent of the soil bacteria died within the first minute when placed in lake water although 9 per cent were still alive after six hours' exposure. A larger proportion of soil bacteria were halotolerant than of other freshwater bacteria.

TABLE 4

Relative numbers of bacteria from the oral cavity which were viable after exposure to Great Salt Lake water (L.W.) of different dilutions for various periods of time expressed as ratios to the number recovered from "formula C" expressed as 100

TIME	"FORMULA C"	LAKE WATER	75 PER CENT L.W.	50 PER CENT L.W.	25 PER CENT L.W.	10 PER CENT L.W.	5 PER CENT L.W.	DISTILLED WATER
1 minute	100.0	6.7	9.8	40.3	73.1	91.5	94.8	102.4
15 minutes	94.6	4.4	10.1	32.6	63.8	80.2	82.3	94.8
30 minutes	95.9	1.7	7.9	29.4	58.4	75.7	84.9	68.5
1 hour	98.2	0.7	5.2	21.0	60.7	72.2	80.6	57.2
2 hours	101.3	0.4	3.3	14.8	51.0	59.6	67.5	51.6
6 hours	109.5	0.2	2.5	6.8	46.2	53.8	72.3	75.8

The death rate of freshwater bacteria is virtually the same in 25 per cent lake water as in 8 per cent sodium chloride,—solutions which are nearly isotonic. Likewise their death rates were the same in more concentrated isotonic solutions of lake water and salt. However, the bacteria survive longer in dilution waters consisting of 10 per cent or less of lake water than in isotonic solutions of sodium chloride. This is attributed to the favorable effect of the mineral balance offered by the dilute lake water whereas in the more concentrated solutions the high osmotic pressure or a specific effect of ions is injurious regardless of the mineral balance. The literature on the favorable effect of balanced salt solutions on bacterial viability is reviewed by Zeug (1920) and Falk (1923).

After the first few minutes both 5 and 10 per cent lake water were less injurious to freshwater bacteria than pure distilled water. In fact, concentrations of lake water ranging from 0.1 to 1.0 per cent compared favorably with "formula C" and in some cases even excelled it for maintaining the viability of freshwater bacteria for extended periods of time. This is not so surprising when one considers that "formula C" is based upon the average composition of river water, and Great Salt Lake water is essentially concentrated river water. Samples of lake water collected in December 1935 had the following composition expressed as grams per liter:

Total salts	336.23 grams
Chloride and other halides	186.29 grams
Sodium and potassium	120.06 grams
Sulfate	19.44 grams
Magnesium	8 32 grams
Calcium	0.40 gram
Carbonate and bicarbonate	0.06 gram

Traces of iron, phosphate, nitrate and ammonium were also detected. The hydrogen-ion concentration was pH 8.0 to 8.3 as determined colorimetrically and corrected for salt error (Parsons and Douglas, 1926). The presence of 0.61 mgm. of total iodine per liter may be significant.

CONCLUSIONS

Further evidence is presented of a bacterial flora indigenous to the Great Salt Lake in northern Utah. From water having a salinity of 27.6 per cent an average of 167 bacteria per milliliter were cultivated on nutrient lake-water agar. Most of the lake bacteria are obligate halophiles whose growth requires at least 13 per cent salt as indicated by the fact that diluting lake water more than 50 per cent inhibits their multiplication. Conversely, few or no bacteria from sewage, soil or the oral cavity grow on lake water media and even 10 per cent lake water inhibits the multiplication of over three-fourths of the freshwater bacteria. Lake water is likewise bactericidal for freshwater bacteria including *Escherichia coli*, *Staphylococcus albus* and the mixed micro-

flora of sewage, soil and the human mouth. Marine bacteria which are generally regarded as halotolerant are killed by a few minutes' exposure to Great Salt Lake water. Besides their halophilic properties, lake bacteria differ from those from other habitats in the slowness with which they develop and the smallness of their colonies. It is believed that they are species which have become acclimatized to the increasing salt concentrations during the time the water of old Lake Bonneville has evaporated leaving its saline remnant, Great Salt Lake.

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STREPTOCOCCUS SALIVARIUS

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Received for publication August 17, 1936

During the past twenty years, with the application of improved methods and more extensive tests, a number of the natural groups or species of the streptococci have become fairly clearly defined. Among the more adequately described groups are those typified by *Streptococcus pyogenes*, the "animal pyogenes," *Streptococcus equi*, *Streptococcus mastitidis*, *Streptococcus lactis*, *Streptococcus fecalis*, *Streptococcus liquefaciens*, *Streptococcus zymogenes*, *Streptococcus bovis* and *Streptococcus thermophilus*. On the other hand, some of the streptococci which have been well known since the relatively early days of bacteriology have not been extensively studied by the more modern methods, and the differentiation of these forms from some of the better established groups is not entirely clear. In such a state are the characteristic non-hemolytic streptococci of the human throat, commonly designated as the "viridans group," *Streptococcus mitior*, *Streptococcus salivarius*, *Streptococcus mitis*, etc.

The present study was limited to the predominating streptococci in the throats of healthy and presumably normal persons. The isolations were made by the application of quantitative dilution methods to freshly taken swab samples, so as to eliminate not only organisms of fortuitous occurrence, but also even streptococci native to the throat which may occur in small numbers. Under these quantitative restrictions true beta-hemolytic streptococci were not obtained among the 322 cultures which were isolated at various times from 20 healthy individuals.

METHODS

The material was collected from the throats (not the mouths) of supposedly normal individuals by means of sterile cotton swabs. Serial dilutions of the material obtained were made in broth, from which poured agar plates were prepared immediately. The agar medium used contained the following nutrients: lactose, 1 per cent; glucose, 0.1 per cent; beef extract, 0.3 per cent; and 0.5 per cent each of yeast extract and peptone. Only well-isolated colonies were subcultured from the plates and all cultures were thoroughly checked for purity before adding to the collection.

Action on blood in poured agar plates was determined on sub-surface colonies, the methods recommended by Brown (1919) being faithfully followed.

The temperature limits of growth were determined in litmus milk, the cultures being adjusted to incubation temperature in a water bath immediately after inoculation and incubation carried out in sealed tubes in accurately controlled incubators. Thermal resistance tests were made in milk. Ten cubic centimeters of sterile skimmed milk were added to 1 cc. of culture and the heavily seeded mixture heated in a sealed tube in a water bath. Tests for ammonia production were made on cultures incubated for one week at 37°C. in 4 per cent peptone solution. Action on starch was determined by plating in proper dilution (not streaking) in starch agar, the plates being flooded with iodine solution after incubation for three days at 37°C.

Substances for the fermentation tests were sterilized separately in 10 per cent solutions and added to sterile yeast-extract peptone broth so as to give a 1 per cent concentration of the test substance. Maltose, inulin and salicin were sterilized by filtration, while the other test substances were autoclaved.

Certain other tests which have distinct value in the differentiation of particular groups of streptococci—such as the pentose sugars and sorbitol (Orla-Jensen, 1919), trehalose (Edwards, 1932), esculin (Meyer and Schönfeld, 1926), inhibition by methylene blue (Sherman and Albus, 1918), and salt tolerance (Sherman and Stark, 1934)—were not used in the present study

because of lack of presumptive relevance. Since the group under study was found to be neatly differentiated from the better established species on the basis of the methods used, it was not necessary to utilize those and other tests which might have been called upon.

THE CHARACTERISTICS OF STREPTOCOCCUS SALIVARIUS

Of the 322 cultures studied, 290 (group I) represented a remarkably homogeneous group. The remaining 32 cultures (groups II, III, IV, and V) showed some variation from the type, but hardly sufficient, we believe, to warrant designation as distinct species at the present time.

Group I (290 cultures)

The typical throat streptococcus, represented by 290 cultures, was found to have the following characteristics.

Morphology. The characteristic grouping is in short chains, the length of chains being somewhat longer, as a rule, in broth than in milk cultures. The size of the individual cells in milk cultures is distinctly larger than those of most streptococci; they are plump and give the impression of being very thrifty. The larger size of the cells is less marked and may be scarcely noticeable in broth cultures.

Blood agar. In horse-blood agar the reaction varies from gamma to alpha, "weak alpha" being characteristic. Very few cultures were encountered which could be classified as truly gamma types, while relatively few gave what might be termed a strong or typical alpha reaction.

Temperature limits of growth. No growth takes place at 10°C. nor at 47°C. The maximum temperature for growth is about 45°C., a minority of the cultures being able to grow at this temperature. The maximum growth temperature of this group appears to be, therefore, somewhat higher than for those groups of streptococci represented by *Streptococcus pyogenes*, *S. mastitidis* and *S. lactis*, but definitely lower than for *S. bovis*, *S. thermophilus*, *S. fecalis*, *S. liquefaciens*, *S. zymogenes*, etc. (Sherman and Albus, 1918; Orla-Jensen, 1919; Sherman and Stark, 1931, 1934; Sherman and Wing, 1935; Hansen, 1935.)

Thermal resistance. There is no survival in milk when heated for 30 minutes at 60°C.

Litmus milk. Litmus milk is promptly acidulated and curdled without previous reduction, but with marked though not quite complete reduction after curdling. There is no apparent digestion of casein. The end result of the action of this streptococcus on litmus milk is much like that given by *S. mastitidis*, but the mouth streptococcus is on the average somewhat faster in bringing about coagulation.

Final pH. In glucose broth a final pH of 4.4 to 4.0 is attained. (These limits were not exceeded by the 290 cultures which made up this "typical" group; the relatively few varieties or aberrant strains which gave atypical results are noted below.)

Sodium hippurate. Sodium hippurate is not hydrolyzed.

Ammonia production. Ammonia is not produced from peptone.

Starch. Starch is not hydrolyzed.

Gelatin. There is no liquefaction of gelatin.

Fermentation reactions. Glucose, maltose, lactose and sucrose are fermented. Glycerol and mannitol are not fermented. Raffinose (93 per cent positive), inulin (80 per cent positive), and salicin (95 per cent positive) are usually fermented.

Pathogenicity. There is no apparent pathogenicity for rabbits by intravenous injection nor for white mice injected intraperitoneally. Not all cultures were tested for virulence, but random selections from this group, in addition to representatives from groups II to V, were used. One cubic centimeter amounts of 0.1 per cent glucose broth cultures were employed.

Viability. One of the noticeable characteristics of this organism is its lack of viability in artificial culture media, notwithstanding its rather vigorous growth in such substrates. It is readily lost with infrequent transfers, being, in our experience, rather more delicate in this respect than the pathogenic streptococci, and decidedly more so than the other non-hemolytic types with the exceptions of freshly isolated cultures of *S. thermophilus* and *Streptococcus equinus*.

Habitat. Human mouth and throat.

Group II (19 cultures)

The cultures of this group differ from those in group I in having limiting pH values of 5.2 to 4.9, with a correlated tendency (by no means rigid) toward a stronger or more typical alpha reaction on blood agar. These cultures do not ferment salicin and only a minority of them ferment raffinose or inulin. Obviously these weak acid-producing strains do not act so vigorously in milk, some failing to cause coagulation.

With a number of correlated divergencies from the typical, these cultures have some right to a group or variety status. However, the differences from the type are all "majority" ones, with the exception of the high limiting pH. The final hydrogen-ion concentration, introduced by Ayers, Johnson and Davis (1918), has proved of great value as a *group* characteristic in the streptococci. On the other hand, individual strains which are weak acid producers are found in all species.

Group III (4 cultures)

This small group is distinguished by the production of an alpha prime reaction on blood agar (Brown, 1919). Again associated with a stronger action on blood is a tendency toward weak acid-producing power. Three of the cultures gave final pH values of 5.4 to 5.2, but the correlation was spoiled by the fourth culture which produced a final acidity of pH 4.2. One of the four cultures ferments raffinose and inulin, while none ferments salicin.

Group IV (8 cultures)

This group is composed of eight cultures, all of which produce ammonia from peptone and give final pH values in glucose broth between 5.3 and 4.9. On blood, and on fermentation tests, they agree perfectly with the members of group I. In some respects this group appears to have rather good claims for recognition as a definite variety, but such a contention in the present state of our knowledge would probably be a mistake.

The production of ammonia from peptone was introduced into the study of streptococci by Ayers, Rupp and Mudge (1921)

and constitutes a valuable test which deserves wider usage. Except for the contributions of Ayers and his associates and a number of papers from this laboratory, little has been reported on the production of ammonia by streptococci. Until the method comes into more general use it is probably wise not to emphasize too strongly the possible taxonomic significance of such observations as are here reported.

Group V (1 culture)

The one culture which makes up this "group" does not ferment lactose, and therefore has no visible action on milk. When litmus milk is supplemented by the addition of 2 per cent glucose, however, it causes rapid curdling followed by marked and almost complete reduction of the litmus in a manner entirely characteristic of the "milk reaction" of the cultures in group I. Aside from its inability to ferment lactose, it agrees in all characteristics studied with the members of group I, including the fermentation of raffinose, inulin and salicin.

It would be easy to follow custom and call this organism *Streptococcus equinus*, but from a careful study of it in comparison with the characteristic lactose-non-fermenting streptococci of horse feces, we feel that it is probably an aberrant strain of the *S. salivarius* group, and should not be confused with the true *S. equinus* of Andrewes and Horder (1906). In this connection it should be mentioned that streptococci which fail to ferment lactose have been obtained frequently, and apparently in substantial numbers, from the human throat (Arnold, 1920).

THE STREPTOCOCCUS MITIS OF ANDREWES AND HORDER

The question naturally arises as to whether we should consider as one species only the rather homogeneous collection of cultures contained in group I, or include also the small groups (II to V) as representing variants from the type. Further work may show that more than one true species may be involved, but we feel that in the light of our present knowledge of the streptococci it would be unfortunate to encumber the literature with more names based on rather shaky foundations.

Those who would divide the organisms here studied into more than one species could combine groups II and III to form a type which is usually marked by a higher limiting pH and a stronger or more definitely alpha type of action on blood, and which usually does not ferment raffinose, inulin or salicin. If such a species were established the appropriate name would appear to be *Streptococcus mitis*. Andrewes and Horder (1906) described *S. mitis* as *usually* not clotting milk nor fermenting raffinose, as opposed to *S. salivarius* which *usually* did. They did not claim that *S. mitis* never ferments raffinose, nor that *S. salivarius* always does. In fact, some 15 per cent of the cultures assigned by them to the species *S. mitis* fermented either raffinose or inulin.

The foregoing paragraph has been criticized by a competent reviewer of this manuscript on the ground that Andrewes and Horder's "Type Form" of *S. salivarius* did not ferment salicin, and that some bacteriologists have more recently differentiated *S. salivarius* from *S. mitis* on the basis of the salicin reaction, those types fermenting this substance being regarded as *S. mitis*. From this point of view it would appear that we are reversing the definitions of Andrewes and Horder; we think, however, that our own descriptions agree with those of Andrewes and Horder, and that the reversal of their distinctions is the work of intervening bacteriologists. This point demands some detailed consideration of the data and statements of Andrewes and Horder.

It appears that the emphasis on the salicin reaction in the differentiation of *S. salivarius* from *S. mitis* probably originated in the work of Holman (1916). This investigator classified the streptococci on the basis of their respective reactions on blood, lactose, mannitol, and salicin; he did not use some of the other tests which, we think, Andrewes and Horder considered of greater importance than salicin. These remarks are in no sense a criticism of the admirable work of Holman whose classification is a model of ingenuity, simplicity, and workability.

With reference to *S. salivarius* (pp. 712–713) Andrewes and Horder state: "It clots milk almost always and in its typical form reduces neutral red, though variants occur which fail to do

this. The characteristic fermentation reactions are saccharose, lactose, and raffinose, the last named less constantly than the first two. The glucoside reactions may be added, and rarely inulin." Again (p. 776) they state: "The common positive chemical reactions are clotting of milk, reduction of neutral red, and acid formation with saccharose, lactose, and often raffinose, but not with mannite. Reactions with the glucosides are often added . . . ." In connection with *S. mitis* (p. 712) Andrewes and Horder remark: "It is a short-chained form . . . and it gives a marked acid reaction in milk, though no clotting . . . . Its typical positive reactions on Gordon's tests are saccharose and lactose with or without the glucosides . . . . Such forms may with fair propriety be regarded as variants of Type D (*S. salivarius*) in which the power of clotting milk has been suppressed." It would appear from these quotations that Andrewes and Horder considered the clotting of milk, reducing action on neutral red, and the fermentation of raffinose as the primary distinguishing characteristics between *S. salivarius* and *S. mitis*, and that they regarded salicin as of subsidiary importance.

Of more importance than what Andrewes and Horder say, however, is what their data show. In table 3 (p. 776) are listed 14 varieties of *S. salivarius*, and 12 of them fermented salicin. However, they designate as the "Type Form" one of the two which did not ferment salicin, but which did ferment raffinose, clot milk, and reduce neutral red. "This form is regarded as the type in virtue of its frequency of occurrence in normal saliva, not in disease. We have met with it once only as a pathogenic agent in a case of malignant endocarditis." Still more to the point perhaps are the "Reaction Curves" (p. 853) on streptococci. These present graphically the distribution of fermentation reactions for large numbers of cultures. These curves represent: all streptococci, saprophytic and parasitic; parasitic streptococci; streptococci producing suppuration; streptococci from cases of malignant endocarditis; and human saprophytic streptococci, based on salivary and fecal specimens. *In each group*, salicin was the most frequently fermented test substance with the exceptions of sucrose and lactose. The test substances

included sucrose, lactose, raffinose, inulin, mannitol, salicin, and coniferin. Salicin was fermented by 70 per cent of all the streptococci studied, and by 67 per cent of the "human saprophytic" group from saliva and feces.

It would appear that, "with or without the glucosides," our group I on the bases of strong acid production (low pH and coagulation of milk), rather strong reducing action, and, in general, the fermentation of raffinose, corresponds to Andrewes and Horder's *S. salivarius*; and that our groups II and III correspond to their *S. mitis*, which "forms may with fair propriety be regarded as variants (of *S. salivarius*) in which the power of clotting milk has been suppressed." The confusion, the hazy borderline, and the fact that Andrewes and Horder themselves considered their *S. mitis* type as probably only a variant of *S. salivarius*, add weight to our previously expressed view that an additional species should not be recognized in this general group in the present state of our knowledge.

If a species of *S. mitis* were accepted, the eight cultures which make up our group IV would perhaps have equal claims to recognition. These cultures, while typical of *S. salivarius* on blood and the fermentation tests, produce ammonia from peptone and have high limiting pH values, the two aberrant characteristics being perfectly correlated in the small group. It is needless to add that we do not at present recommend the recognition of this group, either as a species or as a definite variety.

THE RELATION OF STREPTOCOCCUS SALIVARIUS TO OTHER SPECIES

The important point, which we wish to emphasize especially, is that whether the species is restricted to the typical cultures of group I, or expanded to include the variant types, the group is sufficiently homogeneous and clearly defined so that it can be readily differentiated from any of the adequately described species of the streptococci.

It is unnecessary to point out the numerous points which distinguish this group of streptococci from the hemolytic pathogens, the "enterococci" (*S. fecalis* and its relatives), or the

"lactic" group (*S. lactis* and its varieties). From *S. mastitidis* it is markedly differentiated on the bases of sodium hippurate and blood reactions; in the majority of cases also by the fermentation tests; and, by one familiar with the two groups, slight but significant differences are to be noted in morphology and maximum growth temperatures. *S. thermophilus*, which conceivably might be confused with this organism on superficial study, is widely separated by its much higher maximum temperature of growth, a much higher thermal death point, and its inability, as a rule, to ferment maltose. *S. bovis* has a higher thermal death point, a somewhat higher maximum growth temperature, hydrolyzes starch, and ferments arabinose.

The organism which could be most readily confused with the typical human throat streptococcus is that representative of the "bovis group" which occurs characteristically in the bovine mouth, designated by Ayers and Mudge (1923) simply as *S. bovis* variety B, but given the specific name of *Streptococcus inulinaceus* by Orla-Jensen (1919). This organism differs from the typical *S. bovis* in not fermenting arabinose and not hydrolyzing starch, or attacking it very feebly. It practically invariably ferments raffinose and inulin. Even here the differences from the human throat type appear fairly clearly defined in that *S. inulinaceus* has a higher thermal death point and a slightly higher maximum temperature of growth, usually ferments mannitol, and is usually less vigorous in its action on litmus milk from the standpoint of rate of acid production and extent of reduction of the litmus (Sherman and Stark, 1931). It is of interest and significance to note in this connection that in their valuable paper on the streptococci of the bovine mouth, Ayers and Mudge (1923) stated that on the basis of their unfinished studies of the *S. salivarius* group of the human mouth it appeared that these organisms are distinct.

There is practically no doubt that the non-hemolytic streptococcus which Andrewes and Horder (1906) named *Streptococcus salivarius* is the same as the organism here described. Since it is proposed, for the present at least, not to recognize more than one species in this group, it might well be argued that the older

name, *Streptococcus mitior* (Schottmüller, 1903), be used. This, in fact, is the view taken by us in a preliminary report on this group of organisms (Safford and Sherman, 1936). However, from a careful review of Schottmüller's paper it must be admitted that the types of non-hemolytic streptococci with which he worked could not be now identified. Although Schottmüller worked with throat forms, among others, he apparently used the name *S. mitior* in much the same broad and non-specific sense as "*S. viridans*" is used today. The limited description given of his organisms would apply to several of the now clearly defined species of the streptococci.

For additional authority on this point we would quote the Winslows (1908). These authors deplored the fact that Andrewes and Horder did not make use of the older names in the descriptions of some of their species, but stated: "One of their short-chained forms . . . might well have been identified with *Str. mitior* of Schottmüller. Andrewes and Horder, however, preferred to give new names to their types; and since they have done so their names must stand, as they, for the first time, have described streptococcal types with sufficient clearness and detail to make them definitely recognizable." There would appear to be little doubt that *Streptococcus salivarius* is the name which should be retained for this species.

SUMMARY

A study was made of 322 cultures of non-hemolytic streptococci isolated from human throats. The isolations were made by the application of quantitative methods so as to limit the collection to the predominating types.

Streptococcus salivarius was found to be the prevailing type and 290 of the cultures studied are believed to be typical of the species. A full description of the organism is given.

The remaining 32 cultures varied somewhat from the type but scarcely enough to be considered separate species in the light of present knowledge of the streptococci.

Streptococcus salivarius, as herein described, may be readily differentiated from any of the clearly established species of the streptococci.

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STREPTOCOCCUS FECALIS

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Received for publication September 11, 1936

In some respects *Streptococcus fecalis* (Andrewes and Horder, 1906) might be considered one of the better established species of the streptococci, and certainly some of the rather unique characteristics of this organism, or the general group to which it belongs, are commonly known by bacteriologists. However, much concerning this species is not clear. As the fermentation tests have made up the major portion of the criteria used by many investigators of this organism, and since these tests are extremely variable within this particular species, there is considerable confusion concerning the boundaries of the group and whether one or more species are involved. On the one hand, we have those who would give this group of organisms separate generic standing (*Enterococcus*) quite outside of the streptococci, but many of whom would at the same time lump together a rather heterogeneous mixture of fecal streptococci, as if only one species were contained in this special genus. On the other hand, among those who group these organisms as streptococci, are some who would classify as separate species variants within that rather homogeneous group which is more specifically known as *S. fecalis*. There are, therefore, many points on which additional information is needed. And in passing it might be noted that so long as we are fated to see new papers purporting to show the identity of *S. fecalis* and *Streptococcus lactis*, and to read in papers on the enterococcus that this organism probably includes "the '*S. lactis*' of dairy workers," no apology need be made for the presentation of a paper which contributes, however slightly, to a clearer understanding of this group of organisms.

The present study, though not a continuous one, has extended over several years and has covered 434 cultures which have been identified as *S. fecalis*. All of these cultures were tested for their minimum and maximum temperatures of growth, for action on milk and reducing action on litmus, hemolysis of blood, final pH in glucose broth, production of ammonia from peptone, hydrolysis of starch, liquefaction of gelatin, and ability to ferment each of the ten test substances used. All except 128 of the cultures were tested for ability to hydrolyze esculin and sodium hippurate, and for the production of acetyl-methyl-carbinol. The thermal resistance of the organisms was not tested on the entire collection, but this well known characteristic of the group was checked on 132 cultures. The more recently suggested "tolerance tests"—the ability to grow in media of high alkalinity, and in the presence of relatively high concentrations of sodium chloride and methylene blue—were used on only a few of the cultures of this collection, but we have had sufficient experience with these tests on other cultures to feel sure of their value in studies of this group of the streptococci (Sherman and Stark, 1934; Sherman, Stark and Mauer, 1937).

This study is limited to non-hemolytic and non-proteolytic cultures. The hemolytic and proteolytic members of the "enterococcus group"—*Streptococcus zymogenes* and its relatives—are dealt with in another paper.

THE VALUE OF CERTAIN BASIC TESTS

In a number of papers from this laboratory we have emphasized the value of minimum and maximum temperatures of growth in the study of the streptococci. All of our strains of *Streptococcus fecalis* grew at 10°C. and at 45°C. Some cultures were able to grow at 50°C. and all except five grew at 5°C. Indeed, Foter and Rahn (1936) have shown that at least one of the strains in this collection was able to grow at 0°C.

Taking 10°C. and 45°C. as the test temperatures, we have a rather striking combination for the characterization of *S. fecalis* and its relatives in the enterococcus group—*S. zymogenes* and *Streptococcus liquefaciens*. While not the exclusive property of

these organisms, this combination of temperature tolerance for growth is not found among any of the better known and adequately described species of the streptococci. It is not true of any of the following species, or their varieties: *Streptococcus pyogenes*, *Streptococcus mastitidis*, *Streptococcus lactis*, *Streptococcus salivarius*, *Streptococcus bovis*, *Streptococcus inulinaceus*, *Streptococcus equinus*, and *Streptococcus thermophilus* (Sherman and Albus, 1918; Sherman and Stark, 1931 and 1934; Sherman and Wing, 1935; Hansen, 1935; Safford, Sherman and Hodge, 1937; Hodge and Sherman, 1937). As indicated above, other streptococci are known which can grow at both of these temperatures. Sherman and Wing (1935) studied a hemolytic and non-reducing streptococcus from milk powder, and Mauer (1934) found another hemolytic and non-reducing type in feces, which grew both at 10°C. and at 45°C.

In this connection we beg to be pardoned for mentioning a hoary subject: Including many cultures of *S. fecalis* in addition to the 434 used in this study, we have not yet had a strain of this organism which has failed to grow at 45°C., while among a larger number of *S. lactis* we have not encountered one which grows at that temperature. We do not mean to imply that the temperature limits of growth do not vary as do other characteristics of bacteria. In fact, we have recently pointed out the variation which may occur in this respect in certain lactobacilli (Sherman and Hodge, 1936). It so happens, however, that in the streptococci the normal temperature limits are sufficiently different between certain groups to make these tests of paramount importance.

Another important test in the study of streptococci is one for the ability to cause a complete reduction of litmus in milk before curdling. So far as present information goes, the only streptococci which have this property are those of the "enterococcus group" (*S. fecalis*, *S. zymogenes* and *S. liquefaciens*) and those of the "lactic group" (*S. lactis* and its relatives). It is true that *Streptococcus apis* has also been described as having this characteristic, but the work of Hucker (1932) indicates that this organism and *S. liquefaciens* are identical. All of our cultures

of *S. fecalis* gave a prompt and complete reduction of litmus in milk, with the exception, of course, of the narrow zone which is exposed to the air at the surface. Most of the cultures reduced before curdling in the typical way, but in a few the reduction was completed after curdling. In these cultures, however, the reduction was complete and prompt, and could not be confused by an observant worker with the marked, but not quite complete, reduction after curdling which is given by such organisms as *S. salivarius* and *S. mastitidis*.

The production of ammonia from peptone (Ayers, Johnson and Mudge, 1921) is a valuable, though unexploited, characteristic of certain streptococci. All of our cultures were found to produce ammonia. The ability to hydrolyze esculin has been considered a special characteristic of the enterococcus group (Meyer and Schönfeld, 1926), and none of our cultures of *S. fecalis* failed to attack this substance.

What may eventually prove to be highly specific tests for streptococci belonging to the enterococcus group are those for the ability to grow in broth containing 6.5 per cent of sodium chloride, and in broth adjusted to a pH of 9.6 (Sherman and Stark, 1934; Sherman, Stark and Mauer, 1937). Although these tests have been tried on only 24 of the cultures of *S. fecalis* contained in this collection, all of them grew actively. (In making these tests the sodium chloride and the alkali were added to the previously sterilized 0.5 per cent glucose broths immediately before use.)

The ability to grow in skimmed milk containing 0.1 per cent medicinal methylene blue is a property which is limited to the "enterococcus" and "lactic" groups of the streptococci, in so far as present knowledge extends (Sherman, Stark and Mauer, 1937). The much-used and uncritically-applied test of Sherman and Albus (1918), involving more dilute methylene blue, was devised for another purpose and is of course much less inhibitory. *S. fecalis* grows actively in the presence of 0.1 per cent methylene blue.

DIVERSITY OF FERMENTATION REACTIONS

Although the fermentation tests are known to show considerable diversity in different strains of *S. fecalis*, the extent of

this variability within the species has not been fully appreciated. All of the 434 cultures studied by us fermented glucose, maltose, lactose and salicin. On the other hand, fermenting and non-fermenting strains were found with arabinose, sucrose, raffinose, inulin, glycerol and mannitol. Some strains fermented none of these six substances, while a few strains fermented them all; and it might be added that the collection contained a goodly proportion of the theoretically possible 64 strains obtainable with six diverse characteristics. This in itself would indicate that no species differentiation based on fermentation tests would be permissible within the group, but perhaps it is better to be more specific.

The fermentation of mannitol has long been recognized as especially characteristic of *S. fecalis* (Andrewes and Horder, 1906; Winslow and Palmer, 1910; and many subsequent investigators). Our results confirm these findings in a broad sense, but we had 13 strains out of the entire collection which failed to ferment mannitol. That these mannitol-negative strains do not constitute a definite and clear-cut type is shown by the fact that among this small number were fermenting and non-fermenting strains on arabinose, sucrose, raffinose, and glycerol. In this connection it should be recalled that Dible (1921) also found a variant form of the "enterococcus" which failed to ferment mannitol. *S. fecalis* is usually described as not fermenting inulin. This also is generally true, but 11 of our strains fermented this substance; and among these atypical cultures were strains which fermented, and others which did not ferment, arabinose, raffinose, and glycerol. Again, considering the radical fermentative diversity within such a small number of cultures, the recognition of a type which ferments inulin would not be justifiable on the basis of present knowledge. The same is true with glycerol: Among the glycerol-positive and glycerol-negative cultures were strains which fermented, and others which did not ferment arabinose, sucrose, raffinose, inulin, and mannitol.

This emphasis on the diversity of *S. fecalis* in the fermentation tests should not be interpreted as implying that they are without value in the study of the streptococci. In some groups, certain of these tests are of very great importance; and when applied

in their proper subsidiary rôle they have an important function in the characterization of all of the groups within the genus.

THE STREPTOCOCCUS GLYCERINACEUS OF ORLA-JENSEN

Orla-Jensen (1919) has divided the streptococci of this group, which do not liquefy gelatin, into two species, *Streptococcus faecium* and *Streptococcus glycerinaceus*, based principally upon the fermentation of glycerol. The rather slender basis upon which this differentiation rests has been previously noted (Sherman and Stark, 1931). *S. faecium* has been generally recognized as identical with *S. fecalis*, but the name *S. glycerinaceus* has, to some extent, become attached to the literature. So far as can be told from Orla-Jensen's data, *S. glycerinaceus* appears very definitely to belong in the "enterococcus group." From a study of his titration figures, it also appears that a number of his cultures of *S. faecium* fermented glycerol to some extent, though *S. glycerinaceus* caused a more vigorous fermentation of this substance.

In view of what has been said in the preceding section, it seems scarcely necessary to pursue this question further, but since a separate species has been proposed on the basis of glycerol fermentation, and since *S. fecalis* has frequently been described as not fermenting raffinose and always fermenting mannitol, we have subjected our data to detailed analysis. With these three substances, if the fermentation of each of them is an inconstant characteristic within the species, there would be as possibilities eight combinations of characteristics from (raffinose +, mannitol +, glycerol +) to (raffinose −, mannitol −, glycerol −). Our collection contained seven of these eight possible strains. When sucrose is added to these three substances, the possible number of different combinations becomes 16 and our collection contained 12 of the strains. This would appear sufficient to show that the various reactions on these tests are random ones, and that no additional species can be established in the group, based upon the fermentation or non-fermentation of any one of these substances.

One should not take issue lightly with the magnificent work of

Orla-Jensen. The improbable possibility should still be admitted that his organism is something quite outside of the enterococcus group. If this be the case, his species has not yet been properly defined.

THE CHARACTERISTICS OF STREPTOCOCCUS FECALIS

General characteristics

The characteristic grouping is in pairs and to a less extent in short chains. The organism is hardy and grows well in laboratory media. Blood is not hemolyzed, and gelatin is not liquefied. Milk is acidulated and curdled without digestion of the casein.

Characteristics of primary differential value

Growth takes place at 10°C. and at 45°C. At 5°C. only a few cultures fail to grow, while at 50°C. growth may or may not take place but more often does not.

Litmus in milk cultures is completely reduced, and with relatively few exceptions the reduction takes place before the milk is curdled. In the few strains which reduce after curdling, the reduction of the litmus is prompt and complete below the surface layer, and is in marked contrast with the picture produced by those streptococci which cause a fairly strong but not quite complete reduction after curdling.

Growth takes place in media having an initial pH value of 9.6; in the presence of 6.5 per cent of sodium chloride; and in the presence of 0.1 per cent of medicinal methylene blue in skimmed milk.

Characteristics of secondary differential value

Ammonia is produced in 4 per cent peptone; esculin is hydrolyzed; and a heat treatment of 62.8°C. for 30 minutes, in skimmed milk, is survived. In glucose broth final pH values of 4.4 to 4.0 are obtained; starch is not hydrolyzed; while sodium hippurate may or may not be hydrolyzed, as revealed by the conventional test. Acetyl-methyl-carbinol is produced in skimmed milk cultures.

Fermentation characteristics

The fermentation tests are diverse within the species and these characteristics are regarded as of minor importance in this group.

Glucose, maltose, lactose, and salicin are fermented. Arabinose, sucrose, raffinose, inulin, glycerol, and mannitol may or may not be fermented. Inulin, however, is rarely fermented (11 of 434 cultures), while only a few cultures fail to ferment mannitol (13 of 434 cultures).

SUMMARY

A study was made of 434 cultures of *Streptococcus fecalis* with the application of a more extensive series of tests than has heretofore been used in its characterization. Especial attention was paid to certain basic tests of primary differential value which are not in general use by students of the streptococci. The fermentation tests are diverse within this species and are regarded as of minor value in its description. On the basis of the more fundamental tests, the species is made up of a very homogeneous group of organisms, and no justification was found for its subdivision on the basis of the fermentation tests.

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STREPTOCOCCUS EQUINUS

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Received for publication October 12, 1936

Andrewes and Horder (1906) discovered as the predominating streptococcus occurring in air a type which did not ferment lactose. As horse dung made up a large part of the organic pollution of the London air of that time, they suspected this material as the source of the organism. An investigation of fresh horse dung confirmed their suspicion, this particular organism being not only the principal streptococcus found but usually making up a majority of the total bacterial flora.

This organism, which they named *Streptococcus equinus*, was described by Andrewes and Horder as being devoid of pathogenic properties, non-hemolytic, and having the following additional characteristics: milk is not coagulated; there is little or no reducing action on neutral red; sucrose, salicin and coniferin are usually fermented; lactose and mannitol are not fermented; raffinose and inulin are not attacked as a rule, but a number of variant types which ferment these substances were found. Andrewes and Horder also pointed out the important fact that *S. equinus* has a high minimum temperature of growth, evidenced by little or no growth in gelatin cultures at 20°C. They considered horse dung the chief source of the organism but thought that it might occur in the intestines of other herbivora. They did not succeed in obtaining it from the intestines of certain carnivora examined—the fox and the stoat.

An excellent early contribution to the subject was the work of Winslow and Palmer (1910) which verified the findings of Andrewes and Horder and reported the finding of *Streptococcus equinus* in the intestines of the cow and of man. Those occurring

in the human intestine were believed to represent a distinct variety, marked by its high fermentative power in glucose broth. Among other relatively early contributors to this subject were Fuller and Armstrong (1913) and Broadhurst (1915).

So far as we are aware, there has been no significant contribution to the knowledge of this organism since the early investigations, and with the passing of the years the name *Streptococcus equinus* has become a convenient "wastebasket" into which have been thrown streptococci that do not ferment lactose. Its "identification" has been simplified in the systems of classification. For example, Blake (1917) classified non-hemolytic streptococci which fail to ferment lactose and mannitol as *S. equinus*; while Holman (1916) used the same three tests with the addition of salicin, those failing to ferment salicin being named *Streptococcus ignavus*.

Streptococci failing to ferment lactose have been reported from many sources, including the mouth, throat, urine and feces of humans. Arnold (1920), for instance, found a large proportion of the streptococci isolated from human throats to be unable to ferment lactose. Also, Floyd and Wolbach (1914) and others have isolated non-hemolytic streptococci from human infections, which did not ferment lactose. It is desirable to know whether these widely distributed organisms are in fact *S. equinus*, or if additional species are involved, or, on the other hand, if many of the organisms which fail to ferment lactose are simply aberrant strains of other species rather than the true *S. equinus* of Andrewes and Horder.

The present investigation was undertaken in order to obtain a more adequate description of *Streptococcus equinus* through the use of the more extensive and modern methods now available for the study of the streptococci. A total of 72 cultures which did not ferment lactose, isolated from 20 samples of fresh horse dung, were studied in detail. As in the experiences of former workers, this type was found to be the predominating organism in the intestinal material from the horse.

The various tests employed are mostly well known procedures in the study of streptococci and all of them have been described

in previous papers. For the fermentation tests, the test materials were sterilized separately in 10 per cent solutions and later added to the sterile broth. Mannitol and glycerol were sterilized by heat, the other test substances by filtration.

The reported characteristics were determined on all of the 72 cultures with the exception of their actions on esculin and fructose. After the investigation had been completed and only 15 of the cultures were available, it seemed desirable to test the remaining cultures on esculin because of the supposed importance of this substance in connection with *Streptococcus fecalis* and its relatives among the "enterococci." At the same time, we tested the remaining cultures for action on fructose. The hexose sugars have not been shown to have differential value in the study of streptococci, but, in this case, galactose was included as having possible interest because the organisms involved do not ferment lactose. The tests conducted with the remaining cultures on fructose showed, as was to be expected, that this sugar is also fermented.

Esculin was found to be acted upon, but slowly. When tested after three days' incubation at 37°C. a number of the cultures gave negative reactions, but these negative cultures all gave positive reactions after ten days. Upon retests made in triplicate, it was again found that some cultures were negative after two days, a few still negative after four days, but all were positive after seven days' incubation.

THE CHARACTERISTICS OF STREPTOCOCCUS EQUINUS

Morphology. The characteristic grouping is in short chains, the chains usually being somewhat longer in broth cultures than in milk. Some cultures form extremely long chains in broth.

Blood. In horse-blood agar the alpha type of reaction is given. The degree varies somewhat with different cultures, some giving a weak, but nevertheless definite, reaction.

Minimum temperature of growth. No growth occurs at 10°C. nor at 15°C. At 21°C. very slow growth takes place, requiring about three weeks to give a slight acidity in glucose-peptone-litmus milk, a medium in which the organism grows with especial vigor.

Maximum temperature of growth. Growth takes place at 45°C. but there is no growth at 48°C. Growth seldom occurs at 47°C. (three of 72 cultures).

Thermal resistance. Only ten of the 72 cultures proved able to withstand a heat treatment of 60°C. for 30 minutes in milk. This suggests a somewhat higher resistance than is possessed by the pathogenic streptococci (Ayers, Johnson and Davis, 1918), but substantially lower than that of the thermoduric streptococci (Sherman and Stark, 1931).

Litmus milk. No visible change is produced in litmus milk.

Litmus milk + 2 per cent glucose. With added glucose, litmus milk is rendered acid but is rarely curdled (four of 72 cultures) and there is little reduction of the litmus.

Final pH. In glucose broth, final pH values of 4.4 to 4.1 are attained.

Sodium hippurate. Sodium hippurate is not hydrolyzed.

Starch. Starch is not hydrolyzed under the conditions of the test used. (This test was considered of interest because in some respects *Streptococcus equinus* appears to be related to *Streptococcus bovis* which hydrolyzes starch actively. In three attempts, using both ordinary nutrient agar and yeast-peptone as bases, no growth was obtained on the starch agar plates.)

Ammonia. Ammonia is not produced in 4 per cent peptone.

Esculin. Esculin is hydrolyzed slowly.

Gelatin. Gelatin is not liquefied. (These tests were conducted at 37°C.)

Fermentation reactions. Glucose, fructose, galactose and maltose are fermented.

Sucrose (66 or 72 cultures) and salicin (63 of 72 cultures) are usually fermented.

Raffinose (four of 72 cultures) and inulin (22 of 72 cultures) are usually not fermented.

Arabinose, xylose, lactose, mannitol and glycerol are not fermented.

Viability. *Streptococcus equinus* is readily lost from artificial cultures with infrequent transfers, ranking in this respect with freshly isolated cultures of *Streptococcus thermophilus* and *Strepto-*

coccus salivarius. In this connection, however, it should be recalled that Andrewes and Horder showed *S. equinus* to be very resistant to drying.

DISCUSSION

It is believed that the somewhat extended description of *Streptococcus equinus* here presented shows it to constitute a fairly clearly defined species quite aside from its failure to ferment lactose. Its very high minimum temperature of growth and its high maximum temperature of growth, combined with a relatively low thermal resistance; its feeble action in 2 per cent glucose litmus milk, together with little reducing action; its inability to hydrolyze sodium hippurate or starch, produce ammonia from peptone, or ferment arabinose, xylose, glycerol or mannitol; these, with its general pattern of reactions with other tests, mark fairly clearly the natural boundaries of the species. The variations noted in the fermentation of sucrose, raffinose, inulin and salicin correspond exactly with the findings of Andrewes and Horder. We agree with Andrewes and Horder that there is as yet no sound basis for the division of this group into additional species.

It is probably unnecessary to labor the point, but since divisions of this group on the basis of certain fermentation tests have been suggested, a few further remarks may be pertinent. There were nine cultures which were atypical on salicin, six on sucrose, four on raffinose, and 22 on inulin. In the case of inulin the variant cultures constituted nearly one-third of the collection, but among these strains there were none which were atypical on sucrose or raffinose and three which were atypical on salicin, almost precisely what would be expected from random distribution. Since salicin has been suggested as a differential test, it may be noted that among the variants on this substance none were atypical on sucrose, two on raffinose, and three on inulin. Of the atypical cultures on sucrose none were variant in other respects, except one that fermented raffinose. The only possible correlation which could be considered abnormal from the standpoint of chance distribution, was in the case of two cultures

which were among the four raffinose fermenters, and also among the nine which did not ferment salicin. It would be humorous to suggest these two cultures as constituting a type, but, even if this were done, it would leave remaining 50 per cent of raffinose-fermenting cultures, and 77 per cent of those that failed to ferment salicin, which were not otherwise atypical.

As was previously noted, Holman classified the salicin-negative strains as a new species, *Streptococcus ignavus*. Since Andrewes and Horder defined *S. equinus* as sometimes failing to ferment salicin, such a departure would hardly appear justified in the absence of other correlating characteristics. Bergey (1934) adopts Andrewes and Horder's *S. equinus* and Holman's *S. ignavus* but violates the authors' descriptions in both cases. Aside from the salicin reaction, he describes *S. equinus* as not fermenting raffinose or inulin, while the ability to ferment these substances is ascribed to *S. ignavus*. Andrewes and Horder showed that *S. equinus* sometimes ferments raffinose or inulin, while Holman made no use of raffinose and only one of his 71 cultures of *S. ignavus* fermented inulin. It should be recalled that Andrewes and Horder reported a total of 95 atypical cultures of *S. equinus*, isolated from various sources, which fermented either raffinose or inulin; and that Holman states that *S. ignavus* was so named because of its lack of action on the test substances used.

We do not mean to imply that there is only one species among the non-hemolytic streptococci which do not ferment lactose; only that additional species have not yet been clearly defined. In fact, it would appear from the literature that a detailed study of those organisms obtained from such sources as the human throat and human infections might prove well worth while.

Related to this general problem is the question of how frequently aberrant strains, which do not ferment lactose, occur among the ordinary streptococci. Such are well known to occur even in a strong lactose-fermenting type like *Bacterium coli*, and numerous cultures of *Streptococcus pyogenes*, or cultures that have been so classified, have been reported which lacked this ability. In this laboratory, we have recently had atypical strains of *Streptococcus lactis* and *Streptococcus salivarius* which did not

ferment lactose, but which could be clearly differentiated from *S. equinus*.

Another question is whether or not atypical forms of *S. equinus* which do ferment lactose are of frequent occurrence. We have made no special study to determine this point. It may be pertinent to record, however, that in making this collection 20 lactose-fermenting cultures of streptococci were obtained from fresh horse feces. Eighteen of these were definitely identified as *S. fecalis*, but the other two cultures, though not *S. fecalis*, were not sufficiently studied to allow conclusions concerning their species identity.

It is hoped that the present work may prove of some value in giving a more extensive description of *Streptococcus equinus* and in defining slightly better the boundaries of the species.

SUMMARY

A detailed study was made of 72 cultures of *Streptococcus equinus* isolated from fresh horse feces. The cultures were studied by the application of most of the newer methods which have been found of value in the differentiation of streptococci, in addition to the fermentation tests. It is believed that the data obtained will serve to indicate fairly clearly the natural boundaries of the species.

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AN OPEN SYSTEM RESPIROMETER FOR STUDY OF THE GASEOUS METABOLISM OF MICROÖRGANISMS

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Received for publication September 18, 1936

INTRODUCTION

Most studies of the gaseous metabolism of microörganisms have been performed with manometric respirometers. These respirometers fall into three main groups: (1) the pressure is maintained at a constant, and the change in volume is read directly, as with the Winterstein microrespirometer; (2) the volume is kept constant, and pressure changes are read on a manometer, as in the Warburg form; (3) both pressure and volume change, as in the differential Barcroft type. The closed system type of apparatus has been developed mainly by Haldane (1902), Barcroft (1908), and Warburg (1919). Novy, Roehm and Soule (1925) gave a complete description of their compensator manometer but stated that, "Given an aerobic organism in a sealed tube, it is only a matter of a few hours, as a rule, for the oxygen to disappear and to be replaced by a definite amount of carbonic acid." They also brought out the possibility of the effects of the carbon dioxide and other volatile substances (acetaldehyde, hydrogen peroxide, etc.) on both metabolism and morphology of microbes. Winslow, Walker, and Sutermeister (1932) made additional fundamental observations and Walker, Winslow, and Mooney (1934) showed that the rate of production of CO_2 by *Escherichia coli* in peptone water was much greater when air was bubbled through the culture than when N_2 was used. Dixon (1934) remarked that "All the methods (manometric) assume that no gas other than O_2 and CO_2 is produced or

absorbed. This is generally true except in the case of bacteria. Certain bacteria, however, are liable to give off hydrogen and other gases and in these cases the methods fail." Howland and Bernstein (1931), and Fenn (1935) have endeavored to obviate certain difficulties by various alterations.

Harvey (1928) and Shoup (1929) studied the respiration of luminescent bacteria by both colorimetric and manometric methods. This luminescence is dependent on the dissolved oxygen and oxygen consumption is measured by determination of the time necessary for a suspension of these bacteria in sea water to exhaust the dissolved oxygen to a point where luminescence dims. Harvey found that the dimming point represented the utilization of at least 99.5 per cent of dissolved oxygen and the concentration of oxygen decreased throughout the experiment.

An ingenious method of studying gaseous exchanges of tissues was described by Bennet-Clark (1930). The consumed oxygen is replaced electrolytically, and the quantity of oxygen used is determined by measuring the current utilized in electrolysis. Here the oxygen concentration may be kept more nearly constant than in the manometric respirometers, but again the production of gases other than carbon dioxide may produce errors in the apparent oxygen consumption. Walker (1932) presented a good open system aeration train with which the carbon dioxide and ammonia output of cultures may be studied, but no means for studying oxygen consumption was provided.

PURPOSE

The purpose of the present work then is the development of an open system respirometer with certain points in mind.

(1) To be able to measure oxygen intake and carbon dioxide output of microbic cultures by a direct method as contrasted with the indirect method of pressure changes. The production of volatile by-products other than carbon dioxide will create no source of error.

(2) To avoid excessive accumulation of gaseous by-products of bacterial metabolism.

(3) The apparatus should be adaptable to the observation of

certain phenomena: (a) Changes in cell count; (b) effects of changes in culture media and other environmental conditions upon metabolism; (c) the oxygen usage and carbon dioxide production per cell per unit of time.

GENERAL PLAN OF APPARATUS

The apparatus consists of five major parts:

(1) Air-flow stabilizers which maintain gas-flow at a constant rate, and flowmeters which measure quantitatively the volume of air which passes through the system.
(2) Unit for sterilization of the air which is in train.
(3) Culture unit in which the aerated culture is growing.
(4) Carbon dioxide absorbers.
(5) Oxygen absorbers.

PRINCIPLE OF THE APPARATUS

The principle of the apparatus is as follows: There are two duplicate aeration trains. The culture chamber in one train (Test) contains 150 cc. of medium heavily inoculated with the organism under observation; whereas in the second train (Control) an equal volume of uninoculated sterile medium occupies the flask. The air is passed through the two systems at similar (but not necessarily equal) rates measured by the two flowmeters. In the control system the carbon dioxide and oxygen of the air is absorbed, while in the test train the carbon dioxide and oxygen of the air plus or minus any alterations which may be brought about by the growing culture are determined. Knowing the rate of air-flow, and the interval of aeration, it is possible easily to determine the grams of carbon dioxide and oxygen per cubic centimeter of air in each case. By the composition of the air coming from the control in comparison with that from the test, one may determine the total amount of oxygen used and the carbon dioxide production per cell per hour may be calculated.

AIR-FLOW STABILIZERS

To assure a minimum of variation in the rate of air flow during an experiment, not only was a delicate needle valve used, but also several simple stabilizers were made. See *A* in figure 1.

An elongated glass tube of 6 mm. diameter is placed in a tube of 2 cm. diameter and 75 to 100 cm. long. In this larger tube is placed sufficient dibutyl phthalate for a column 50 or 75 cm. high. The air passes from the stabilizers to the flowmeters at a constant pressure as long as an excess of air is escaping from the lower, inner end of the small tube of *A*, figure 1. The pressure is equal to the weight of the column of liquid displaced by the air in bubbling out at that orifice. Thus, pressure necessary for the gas to pass through the remainder of the aeration train may be

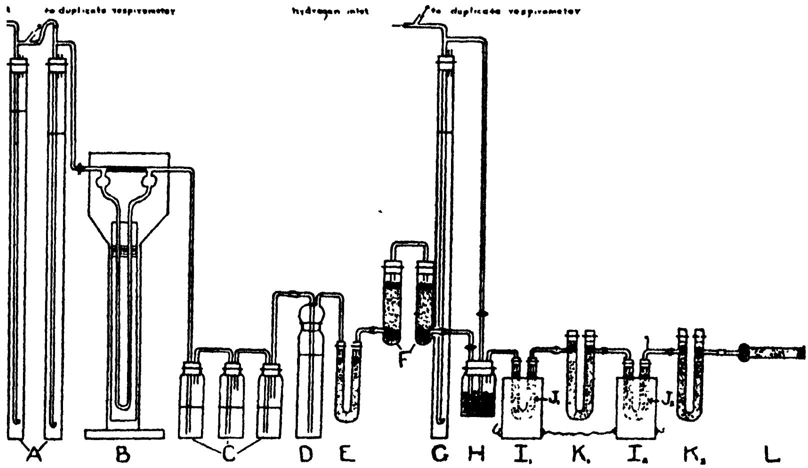

FIG. 1. PLAN OF OPEN SYSTEM RESPIROMETER—OPEN TRAIN RESPIROMETER

A, airflow stabilizers; *B*, flowmeter; *C*, sterilizing unit; *D*, culture chamber; *E*, drying tube; *F*, ascarite tubes; *G*, hydrogen stabilizer; *H*, gas mixing chamber; I_1, first heater; I_2, second heater; J_1, first catalyzing chamber; J_2, second catalyzing chamber; K_1, first weighed drying tube; K_2, second weighed drying tube; *L*, auxiliary drying tube.

attained by adjusting the depth of the dibutyl phthalate. The excess air escapes through a vent at the top of the large tube.

One stabilizer with outside tube 110 cm. long and containing a column of liquid 90 cm. deep is connected with heavy tubing to the tank containing the compressed air. The gas coming from this first stabilizer is divided by means of a T tube and passes into two additional stabilizers of the same design except that the outside tube is 75 cm. long, and the column of dibutyl phthalate is 50 cm. high. The air from one of these last two stabilizers

passes into the test system, whereas the air from the other stabilizer passes into the control series.

FLOWMETERS

The drop in air pressure noted by the difference in levels in fluid in the two sides of a flowmeter is a function of the diameter and length of the capillary and of the rate of air flow. By calibrating the flowmeters it is possible to determine accurately the rate at which air is passing through this unit.

The capillary tube utilized in our experiments was 0.6 mm. diameter (inside) and approximately 7.5 cm. long. The diameter and length of the capillary used may be varied according to the maximum and minimum rates of flow desired. The above dimensions give satisfactory results for rates of gas flow lying between 10 and 100 cc. per minute. The U tube of the flowmeter was made of barometer tubing having an inside diameter of 3 mm. The fluid used in the U tubes was dibutyl phthalate plus a small amount of anilin red to color it. The flowmeter is incorporated in figure 1 as *B*.

STERILIZATION UNIT

The air is sterilized by passing through 150 cc. of 10 per cent H_2SO_4; then it is washed in 150 cc. of distilled water, and finally is passed through sterile non absorbent cotton. See *C* of figure 1.

CULTURE CHAMBERS

The culture chambers are Drechsel gas-wash-bottles of 250 cc. capacity with ground glass fittings and are included as *D* in figure 1. One hundred fifty cubic centimeters of medium (sterile in control; inoculated in test) are placed in each chamber. The air bubbles through the culture, thus supplying oxygen and driving the carbon dioxide or other volatile by-products out of the culture chamber.

CARBON DIOXIDE ABSORBERS

The gas is dried by passing from the culture unit through a U tube containing barium perchlorate which salt is used because

its vapor pressure is very close to that of NaOH. The gas is then passed into ascarite tubes to remove all CO_2, as described by Buck (1926). The ascarite tube is made by sealing a side arm on to a pyrex test-tube 1 cm. from its bottom. The side arms in the present work were made 5 cm. long and consisted of the inner cone of a No. 7 standard pyrex replaceable ground-glass connection. In both the control and test systems two such tubes were used in a series so that the second tube would catch any carbon dioxide which might pass the first tube. The gain in weight in the second tube has always been found to be negligible. These tubes are *E* and *F* in figure 1.

OXYGEN ABSORBERS

The CO_2-free air leaving the second Ascarite tube passes into a small gas-mixing chamber, *H* in figure 1. This chamber consists of a low, wide-mouthed bottle of 75 to 100 cc. capacity. The bottle is half filled with clean glass beads, and sufficient concentrated sulphuric acid to give a column of 2.5 cm. depth. The CO_2-free air is mixed with hydrogen in this chamber. Each gas has a separate entrance tube and controlling stopcock. In this fashion both gases may be introduced at the same time or either one may be stopped while the other continues to flow. The sulphuric acid is employed primarily to fill a twofold need: (1) to act as a trap so that the hydrogen is kept from entering the air line; (2) to provide an excess of hydrogen which, as will be shown later, is necessary. By watching the rate of bubbling of the hydrogen through the acid, it is easy to judge whether the necessary excess of hydrogen is being introduced. The rate of hydrogen flow is maintained at a constant in the two systems by means of two additional stabilizers such as have been described previously—*G* in figure 1. The sulphuric acid also aids in drying both the hydrogen and the CO_2-free air, while the turbulence caused by the glass beads ensures a thorough mixing of the two gases.

The gas mixture is then completely dried by passing through a U tube containing barium perchlorate, after which the dried gases pass into a U tube containing 2 grams of palladinized asbestos, I_1 and I_2 of figure 1. The palladium catalyzes the re-

action between hydrogen and oxygen to form H_2O. Either 5 or 10 per cent commercial palladinized asbestos was found to be satisfactory. The moisture thus formed is driven into a weighed drying tube containing barium perchlorate and inserted at K_1 and K_2 in figure 1. From this the air passes through a second similar catalyzing chamber and a second weighed drying tube. This second catalyzing chamber was found to be necessary because, when the rate of air-flow is 20 to 25 cc. per minute, as much as 5 per cent of the oxygen in the air remains uncombined after passing through the first tube (I_1). A third tube was not found necessary. A small electric heater is placed under each catalyzing tube. By heating the air around the catalyzing tubes to approximately 250°C. several benefits are derived: (1) the passage of the water from the catalyzing tube into the drying tubes is hastened; (2) the catalytic action is much more complete at this temperature than at that of the room; (3) the palladinized asbestos appears not to become "poisoned" at this temperature. The method then is gravimetric.

The U tubes containing the palladinized asbestos and the weighed drying tubes were made of 11 mm. pyrex glass tubing. The side-arms were fabricated from the inner cones of No. 7 ground glass connections as in the case of the ascarite tubes and the outer cones of the ground glass connections were attached to the U tubes containing the catalyzer. Thus, all parts which had to be removed from the aeration train and weighed (e.g., drying and ascarite tubes) were connected to the train in their proper places by means of standard ground glass connections. These connections were found to be air-tight without the use of grease or lubricant at pressures far greater than those which ever existed in the apparatus. The ascarite tubes when charged weighed approximately 32 grams, and the drying tubes 45 grams.

EXPERIMENTAL STUDIES

Before an experiment could be begun it was necessary to remove all moisture and oxygen from the catalyzing chambers. This was accomplished by driving dry hydrogen through the catalyzing and drying series. This is done in train, unit by unit.

Preliminary to metabolic studies it was necessary first to prove

that the described aeration train could analyze accurately a flowing stream of air. In the first series of experiments, then, no cultures were used. One hundred fifty cubic centimeters sterile medium of the composition to be used in the later work were placed in both culture chambers. Air was bubbled through both systems at constant similar rates measured by the flowmeters. The duration of the average experiment was two hours. The total water and carbon dioxide absorbed in each system was determined. The rate of flow and the time interval of flowing was known; therefore, the total number of cubic centimeters of air passing through each system was easily computed. Then, comparisons were made of the grams of carbon dioxide and oxygen per cubic centimeter of air as determined in successive experiments. The following is a sample calculation:

Experiment number = Series I, 1.
Rate of air flow = 11.9 cc. per minute.
Time interval = 120 minutes.
Grams H_2O absorbed = 0.5907.

$$\frac{0.5907}{11.9\text{x}120} \times \frac{32^*}{36} = 3.68 \times 10^{-4} \text{ grams } O_2 \text{ per cubic centimeter air.}$$

* 32/36 = conversion factor H_2O to O_2.

During an experiment the hydrogen must be flowing at a rate equal to or a little greater than that of the air in order that there may be available an excess of hydrogen in the catalyzing chambers. Only one-fifth of the air consists of oxygen, and the use of hydrogen must be governed accordingly.

In order to have a supply of air which should be constant in composition for a number of experiments, air was compressed in a tank to a pressure of 75 to 80 pounds per square inch.

Since there are many satisfactory methods of absorbing quantitatively the carbon dioxide from a flowing stream of air, this factor was considered of less importance in the present work. The chief emphasis has been placed upon developing a method of determining accurately the amount of oxygen in a moving body of air.

In table 1 are the results obtained for grams of O_2 per cubic

centimeter of air using the described apparatus. There were conducted three series of experiments which were alike except that a new supply of air had been placed in the tank for each series. Since the amount of CO_2 per cubic centimeter of air is so negligible (approximately 5.4×10^{-7} grams per cubic centimeter at atmospheric pressure and room temperature) there were only slight increases in the weights of the ascarite tubes and these were well within the limits of experimental error of weighing. Therefore the CO_2 results are omitted.

TABLE 1

EXPERIMENT NUMBER	RATE OF AIR-FLOW	TIME INTERVAL FLOWING	WATER ABSORBED	EQUIVALENT OXYGEN PER CUBIC CENTIMETER OF AIR
	cc. per minute	*minutes*	*grams*	*grams*
Series I:				
X	11.9	120	0 5907	$3\ 68 \times 10^{-4}$
XI	15.8	120	0.7827	$3\ 67 \times 10^{-4}$
XII	19 8	120	1 0248	$3\ 75 \times 10^{-4}$
Average..				$3\ 70 \times 10^{-4}$
Series II:				
XI	10.6	120	0.4648	$3\ 25 \times 10^{-4}$
XIII	14 4	120	0 6252	$3\ 22 \times 10^{-4}$
Average..				$3\ 24 \times 10^{-4}$
Series III:				
XIV a	21 4	106	0 8071	$3\ 16 \times 10^{-4}$
b	19 2	97	0 6683	$3\ 19 \times 10^{-4}$
XV a	18 6	123	0 8120	$3\ 16 \times 10^{-4}$
b	23 8	123	1 0195	$3\ 10 \times 10^{-4}$
Average...				$3\ 15 \times 10^{-4}$

In the above results, it is evident that oxygen in a flowing stream of air may be determined with accuracy by the method employed. Results in both trains of the apparatus were closely similar.

It remained then to show that this respirometer not only gave constant results for the oxygen composition of a given body of air but that it could measure the oxygen usage and the carbon

dioxide production of a microbic culture. To do this a number of experiments were run, using *Saccharomyces cerevisiae* as the organism to be studied.

PROCEDURE IN THE METABOLIC STUDIES

The yeast was grown on a large slant of Sabouraud's agar for several days at 27°C. Forty-eight hours previous to the experiment, 10 cc. of liquid medium in which the yeast was to be growing during the run was added to the slant, and the culture was incubated for an additional 24 hours. The heavy growth thus obtained in the 10 cc. of medium then was poured into the culture chamber of the apparatus, 140 cc. of fresh medium were added and incubated at 27°C. until the time of the experiment. In the first two of our experiments, this final incubation period was 36 hours but in all others it was 24 hours. Two hours before the actual run was begun, the culture chamber was removed from the incubator and connected into the aeration train. Air was bubbled through the culture chamber in order to drive out the accumulated carbon dioxide and to bring the liquid to equilibrium with the external atmosphere. The experiment itself usually continued for approximately two hours and except for the fact that a growing culture was occupying one of the culture chambers, the procedure was the same as that described previously. The air was bubbled through both systems at measured rates. Since the rates of flow do not remain entirely constant throughout a run, flowmeter readings were taken every minute until both trains of the respirometer had come to equilibrium, and then every ten minutes until the end of the test. At the same time the H_2-flow was adjusted to supply an excess. The air then was turned off and hydrogen continued to pass through the catalyzing chambers until all the water produced from the hydrogen and oxygen was driven over into the drying tubes.

Immediately before and after each run, Sabouraud's agar pour plates were made of the culture. The cell counts always were found to be increased only slightly after the two-hour period. Therefore, to determine the average number of living cells present during the experiment, the arithmetical mean of the "before" and

"after" counts was computed. It was possible to obtain counts which checked satisfactorily if care was taken with mixing dilutions. The pour plates were made in quadruplicate of the cultures diluted to 10^{-5}, 10^{-6}, and 10^{-7}. The plates were incubated at 27°C. for 48 hours before counting.

After the experimental run was completed, and when all of the water was driven from the catalyzing chambers into the drying tubes, the ascarite and the drying tubes were carefully stoppered and rubber policemen were fitted over the side arms. These tubes then were placed in a desiccator for several hours before the weighings were made in order to remove any moisture from external surfaces.

TABLE 2

EXPERIMENT NUMBER	MOLS O_2 USED BY CULTURE PER HOUR	AVERAGE NUMBER OF LIVING YEAST CELLS PRESENT	MOLS O_2 USED PER CELL PER HOUR	MOLS CO_2 PRODUCED PER CELL PER HOUR
XVI*	0.53×10^{-2}	1.78×10^{10}	0.30×10^{-12}	0.16×10^{-12}
XVII*	0.45×10^{-2}	1.75×10^{10}	0.26×10^{-12}	0.21×10^{-12}
Average			0.28×10^{-12}	
XIX	0.24×10^{-2}	1.29×10^{10}	0.19×10^{-12}	0.23×10^{-12}
XX	0.18×10^{-2}	1.02×10^{10}	0.17×10^{-12}	0.28×10^{-12}
XXI	0.21×10^{-2}	1.09×10^{10}	0.20×10^{-12}	0.30×10^{-12}
XXIII	0.11×10^{-2}	0.673×10^{10}	0.16×10^{-12}	
XXIV	0.10×10^{-2}	0.530×10^{10}	0.18×10^{-12}	
Average			0.18×10^{-12}	

* Final incubation period of 36 hours duration; all others, 24 hours.

Table 2 is a composite of the results obtained in the present studies. As mentioned earlier, chief emphasis has been placed upon the oxygen usage since there are many satisfactory methods of studying carbon dioxide production. Consequently, a particular precaution which must be taken for the accurate study of carbon dioxide production, but which does not affect the study of oxygen consumption, was ignored here. This was an analysis of the culture medium before and after each experiment for "bound" carbon dioxide (carbonates).

The apparent high carbon dioxide production per cell per hour

may be explained upon the basis that the yeast was growing in a medium rich in glucose content, and anaerobic fermentation may have been proceeding at the same time as oxidation. However, since no studies were made of the changes in composition of the medium in so far as sugar content was concerned, no definite conclusions on this point may be deduced. A point of interest here was that demonstrated by Meyerhof (1925). This author found that one mol of respired oxygen causes the disappearance of 1.5 to 2 mols of fermented carbon dioxide, that is, oxidation of one mol of glucose protects 4 to 6 mols of glucose from being fermented. Meyerhof found further that under aerobic conditions, Brewer's yeast ferments 50 mols of glucose for every mol oxidized; but after 15 hours in a glucose-phosphate solution the ratio drops to 4 mols fermented to 1 oxidized. For pressed yeast this ratio was from 2 to 4 fermented to 1 oxidized, and for wild yeast, 0.3:1. It seems apparent, then, that to report the respiratory quotient merely in terms of carbon dioxide produced and oxygen used, as is done frequently, may be of little significance unless careful studies are made of the simultaneous changes occurring in the chemical composition of the culture medium. Pulley and Greaves (1932) determined that the amount of carbon dioxide produced by yeasts under varying conditions is not proportional to the increase in the number of cells present.

CULTURE MEDIUM

In all of the experiments the medium used was similar to that listed as No. 193 in the compilation of culture media by Levine and Schoenlein (1930). However, glucose was substituted for sucrose, and 0.5 per cent Difco peptone was added. This medium consisted of: water, 1000 cc.; NH_4Cl, 1.88 gram; glucose, 100 grams; K_2HPO_4, 1 gram; peptone, 5 grams. Sterilization was by means of a Berkefeld filter. However, the Arnold steamer may be used.

DISCUSSION

In table 2 it will be noted that the average yeast cell consumes approximately 0.20×10^{-12} mols of oxygen per hour in the rich

medium used in our work. It is apparent that the results for the oxygen consumption in the various experiments check very satisfactorily. The possibility of errors in the cell counts would permit of greater variation.

In spite of the great abundance of studies of yeast metabolism reported in the literature, few papers present the data in such

TABLE 3

ORGANISM	REPORTED BY	EXPERIMENTAL CONDITIONS	MOLS O_2 PER CELL PER HOUR
Lactobacillus pentoaceticus	Hunt (1933)		0.36×10^{-15} to 0.85×10^{-15}
Sarcina lutea	Gerard and Falk (1931)	In water	0.28×10^{-15}
		In glucose	0.28×10^{-15} to 0.84×10^{-13}
Photobacterium phosphorescens	Harvey (1928)	15°C.	0.56×10^{-15}
		21.5°C.	1.33×10^{-15}
Commercial yeast	Field, Martin, and Field (1935)	pH 6.0	0.6×10^{-13}
		pH 6 8	0.7×10^{-13}
Yeast (species?)	Harvey (1928)*		$0\ 85 \times 10^{-13}$
Saccharomyces cerevisiae	Field (1935)	pH 5.8	0.14×10^{-13}
		pH 6 0	$0\ 13 \times 10^{-13}$
		pH 6.8	0.14×10^{-13}
		pH 7 8	$0\ 06 \times 10^{-13}$
Saccharomyces cerevisiae	Author (1936)	Aerated in glucose	0.18×10^{-12}
Arbacia eggs	Tang (1931)	Unfertilized	1.3×10^{-12}
Arbacia eggs	Tang and Gerard (1932)	Fertilized	$4\ 8 \times 10^{-12}$
Paramaecium caudatum	Howland and Bernstein (1931)		2.0×10^{-11}
Actinosphaerium eichornii	Ibid		4.6×10^{-11}

* Harvey cites the given value, but indicates that the author was unknown to him.

form that it is possible to determine the oxygen consumption per yeast cell per hour. In Meyerhof's work, a given weighed sample was used for the inoculum. No cell counts were made. The oxygen consumption was reported in mols of oxygen per mg. dry weight of yeast. From the data given, there is no way to determine the proportion of living and dead cells present. Stier (1933)

when studying the effects of temperature upon oxygen consumption of yeast, stated that standard suspensions were used throughout the work, but failed to indicate how many cells were present. Many other workers report the oxygen consumed per cell per hour in terms of cubic millimeter with no reference to temperature and pressure. Walker (1932), Meyerhof (1925), and others point out that the presence of sugar in the culture medium, and aeration also, increase greatly the rate of oxygen consumption of microorganisms.

To be able, then, to compare the rate of oxygen consumption of yeast as determined by the present method with the rates as measured by other methods available, it would be necessary to use the same strain of organism, culture medium, etc., in all series included. But, since the primary purpose of the present work was to develop a respirometer which approached the optimum more closely, the experiments were not designed specifically for comparison with other work of similar nature. Nevertheless, it may be of interest to compare the rate of oxygen consumption of yeast as determined here with those of other organisms as they have been observed by workers using various types of respirometers. Table 3 affords such an opportunity. In the cases in which the author has reported the amount of oxygen consumed in cubic millimeter per cell per hour, it has been necessary to assume definite conditions of temperature and pressure in order to convert the results into mols of oxygen per cell per hour.

SUMMARY

1. With the described aeration train it is possible to determine the mean rate of oxygen consumption per cell per hour.

2. When *Saccharomyces cerevisiae* is growing in an aerated culture medium rich in glucose, a 24-hour culture utilizes 0.18×10^{-12} mols of oxygen per cell per hour; a 36-hour culture, 0.28×10^{-12} mols.

3. The results for the rate of production of carbon dioxide by yeast did not check in the present work as satisfactorily as did those for oxygen consumption. This may be explained on the basis that:

(a) Only the gaseous carbon dioxide was determined.

(b) Some of the carbon dioxide produced will be found in the "bound" form. To determine this would necessitate a careful chemical analysis of the culture medium before and after the respiratory tests are made.

(c) Much carbon dioxide is produced through enzymatic activity which does not involve any consumption of oxygen. Therefore, in order to interpret respiratory quotients, it is necessary to know what proportion of the sugar consumed during an experiment is fermented and what is oxidized. This may be determined by comparing at least three items: (1) total sugar consumed; (2) total carbon dioxide produced (free and bound); (3) total oxygen consumed.

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FILTRATION OF TREPONEMA PALLIDUM AND TREPONEMA NOVYI THROUGH COLLODION MEMBRANES

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Received for publication September 25, 1936

The ability of the smaller spiral organisms to pass Berkefeld filters was first observed by Novy and Knapp (1906) with *Treponema novyi*. Inada and his co-workers (1916) found that *Leptospira icterohaemorrhagiae* was also filterable. *Treponema pallidum* was found by Noguchi (1911) to grow through the pores of Berkefeld V and N filters in 5 days under favorable cultural conditions but not to pass through by ordinary suction methods. Recently Hindle and Elford (1933), using gradocol membranes as small as 0.4 μ average pore diameter, were able to recover in filtrates the organisms from Kroo's and Vásárhelyi's *Treponema* cultures. They also filtered cultures of *Leptospira icterohaemorrhagiae* and found that a pore diameter of 0.25μ was large enough to pass this organism. They did not filter the treponemas of the relapsing fever group.

In the present experiments suspensions of testicular tissue from syphilitic orchitis of rabbits were used as a source of *Treponema pallidum*, and with gradocol membranes no difficulty has been experienced in recovering the organisms, which were found by direct microscopic examination in the filtrates and were also identified by the production of orchitis in rabbits. The strain used was that isolated by Nichols (1914) in 1913 and was obtained through the courtesy of Dr. Louise Pearce, of the Rockefeller Institute for Medical Research. It has been maintained in this laboratory for 3½ years and has never failed to produce orchitis in rabbits, even when spirochetes were not demonstrable

by darkfield examination, as, for example, in lymph gland material from rabbits which had recovered from orchitis several months previously, or in the brain and spleen of mice with the so-called asymptomatic syphilis following subcutaneous inoculation of syphilitic rabbit tissue. This material was therefore excellent for the present purpose, since the presence of very few spirochetes in a given filtrate could hardly fail of detection by inoculation.

As representative of the relapsing fever type of *Treponema*, we have used *Treponema novyi*, kindly furnished by Dr. W. J. Nungester, of the Hygienic Laboratory, University of Michigan, where the organism has been maintained by rat passage for many years. It had been carried in this laboratory for several months, the infected blood being stored in the icebox between rat passages. Blood taken from a rat at the time of maximum multiplication of the spirochetes, as shown by examinations of tail blood, has never failed to produce infection unless stored longer than 3 weeks.

METHODS

Rabbits infected with *T. pallidum* were usually castrated 1 to 2 weeks after induration of the testicle was first observed, the spirochetes being most numerous at this time. The indurated tissue was ground in approximately 2 volumes of saline in a mortar with sand, rinsed into a centrifuge tube with another volume of saline, and centrifuged at low speed for 10 minutes to throw down the tissue débris. One rabbit was inoculated with the supernatant fluid before filtration and another after filtration. The unfiltered material was allowed to stand until filtration was completed, a period sometimes as long as an hour and a half in the case of the smaller membranes, and the two rabbits were inoculated at the same time. The volume of unfiltered suspension inoculated was 0.3 to 1.0 cc., according to the amount available and the number of spirochetes seen by darkfield examination. The remaining suspension was filtered and the entire filtrate injected. Observations of the rabbits were made weekly, and the orchitis produced by the filtrate was identified by the presence of the treponemas in the lesion and by passage to other rabbits. In some instances the material from a positive filtrate rabbit was used for the next

filtration experiment. Experiments were duplicated for membranes of a given pore size.

The membranes used were prepared according to the technique of Elford (1931), as modified by Bauer and Hughes (1934). Filtration was carried out at room temperature under 50 pounds nitrogen pressure in Bauer and Hughes' filter chamber. The membranes were tested with *Serratia marcescens* and also with a small vibrio obtained from sewage in this laboratory.[1] Membranes of A.P.D. 0.75μ and smaller did not pass *S. marcescens*, but the small vibrio traversed membranes down to A.P.D. 0.46μ.

In the case of the rat blood containing *T. novyi*, filtration was conducted with plasma obtained by means of a pipette after allowing the citrated blood to stand for 24 hours in the icebox. The use of plasma rather than whole blood facilitates filtration. The rats were bled at the time of maximum multiplication of the spirochetes, usually 48 hours after inoculation. The plasma from the control rat, i.e., the one which had received unfiltered blood, was used for the subsequent filtration experiment.

RESULTS

As table 1 shows, *T. pallidum* passed membranes of A.P.D. 0.58μ down to 0.4μ rather readily, while two experiments with a membrane of 0.36μ proved negative (rabbits 8F and 9F). Both animals of the negative experiment were shown to be free from infection by transfer of the popliteal glands to another rabbit, with negative results. They were also reinoculated after removal of the glands with an unfiltered testicular suspension; one died soon after inoculation, the other subsequently developed a typical orchitis. Since abundant spirochetes were present in the suspensions of which filtrates were injected into rabbits 8F and 9F, and the amount of filtrate injected was in the latter instance rather large (1 cc.), it seems probable that *T. pallidum* is unable to pass through a membrane of 0.36μ. This result approximates that of Hindle and Elford with culture treponemas.

The results with *T. novyi* are presented in table 2. The "limiting pore size" proved to be 0.52μ, that is, the organisms require a

[1] Isolated by Dr. C. A. Colwell.

TABLE 1

Filtration of Treponema pallidum

Source of test material—rabbit number	Number of spirochetes before filtration per field	A.P.D. of membrane	Time of filtration	Volume of suspension	Volume of filtrate	Number of spirochetes in filtrate per drop	Result of rabbit inoculation: Material inoculated	Rabbit number	Amount of inoculation	Result	Incubation period	Remarks
		μ	*minutes*	*cc.*	*cc.*				*cc.*		*days*	
316	6–8	0.58	30	3.0	0 5	†	Unfiltered	1	0.5	Died		
							Filtered	1F	0 5	Positive	66	See 3 and 3F
318	4–6	0.58	15	4.0	1.0	6	Unfiltered	2	0 5	Positive	27	
							Filtered	2F	1.0	Positive	71	
1F	3–4	0.46	15	3.0	0 5	0	Unfiltered	3	0.5	Positive	36	See 6 and 6F
							Filtered	3F	0.5	Positive	43	
321	1	0.52	30	3 0	1 8	0	Unfiltered	4	0.65	Positive	28	See 8 and 8F
							Filtered	4F	1.8	Positive	48	
2	3–4	0.58	30	4.0	1.5	0	Unfiltered	5	1.0	Positive	32	
							Filtered	5F	1.5	Positive	40	
3F	12–15	0.40	60	4.0	1.2	2	Unfiltered	6	0.2	Experiment not completed*		
							Filtered	6F	1.0	Note positive microscopic findings		
340	3–4	0.47	90	3.0	1.9	0	Unfiltered	7	0 4	Positive	40	
							Filtered	7F	1.75	Positive	61	

* The animals of this experiment were lost during the writer's prolonged absence from the laboratory, but the experiment is included because microscopic examination of the filtrate was positive, and the membrane used was shown to be intact by its failure to pass either *S. marcescens* or the small vibrio.

† Not examined.

TABLE 1—*Concluded*

SOURCE OF TEST MATERIAL—RABBIT NUMBER	NUMBER OF SPIROCHETES BEFORE FILTRATION PER FIELD	A.P.D. OF MEMBRANE	TIME OF FILTRATION	VOLUME OF SUSPENSION	VOLUME OF FILTRATE	NUMBER OF SPIROCHETES IN FILTRATE PER DROP	RESULT OF RABBIT INOCULATION					
							Material inoculated	Rabbit number	Amount of inoculation	Result	Incubation period	Remarks
		μ	*minutes*	*cc.*	*cc.*				*cc.*		*days*	
4F	3–5	0.36	40	3.0	0.3	0	Unfiltered	8	0.3	Died		
							Filtered	8F	0.3	Negative 180 days. Lymph node transfer 180 days; result negative. Reinoculated 180 days; positive in 34 days		
350	3–4	0.36	90	6.0	1.0		Unfiltered	9	1.0	Positive	41	
							Filtered	9F	1.0	Negative 93 days. Lymph node transfer 93 days; result negative. Reinoculated 93 days, died		

larger pore diameter to pass through, as would be expected from the fact that they measure about 0.3μ microscopically, as compared with 0.2 to 0.25μ for *T. pallidum*. In filtrates from membranes of A.P.D. 0.68μ and 0.74μ the spirochetes were found in considerable numbers and produced infections comparable with that in control rats. The filtrate from the 0.57μ membrane induced definite infection but the overwhelming numbers of spirochetes in the blood which were always found in control rats were not observed.

DISCUSSION

Filtration through Berkefeld candles has been used as a method of obtaining pure cultures of *Leptospira biflexa* from water (Bauer, 1927). In this case the amount of spirochete-containing fluid is unlimited, it contains only a small amount of colloidal material,

and the leptospiras are extremely thin and very vigorously motile. The treponemas in suspensions of testicular tissue do not pass Berkefeld candles readily, partly, perhaps, because of the high concentration of colloidal material, but probably chiefly because

TABLE 2

Filtration of Treponema novyi

A.P.D. OF MEMBRANE	TIME OF FILTRATION	VOLUME OF PLASMA	VOLUME OF FILTRATE	SPIROCHETES IN PLASMA	SPIROCHETES IN FILTRATE	RESULT OF INOCULATION: Material inoculated	Rat number	Amount inoculated	Result	Incubation period
μ	*minutes*	*cc.*	*cc.*					*cc.*		*days*
0.74				++++	++	Unfiltered	1	0 5	Positive	2
						Filtered	1F	2 0	Positive	5
0.68	10	5	4	++++	++	Unfiltered	2	0.8	Positive	3
						Filtered	2F	2.5	Positive	5
0.57				++++	+	Unfiltered	3	0.5	Positive	3
						Filtered	3F	4.0	Positive	5
0.52				++++	0	Unfiltered	4	0.5	Positive	2
						Filtered	4F	2.0	Negative	Examined daily for 7 days
0 52	75	3	0 8	++++	0	Unfiltered	5	0.4	Positive	2
						Filtered	5F	0.8	Negative	Examined daily for 8 days
0.52				++++	0	Unfiltered	6	0.6	Positive	2
						Filtered	6F	0.6	Negative	Examined daily for 8 days

of adsorption on the relatively enormous filtering surface. Novy and Knapp (1906) secured their best results in the filtration of *T. novyi* by shaving their Berkefeld candles to about one-fourth their original thickness. With the collodion membrane adsorption is reduced to the minimum, and if the material to be filtered

is centrifuged at low speed for 10 minutes to remove débris which will block the filter, the volume of the filtrate may be 50% to 75% that of the original suspension in the case of the membranes of larger pore diameter. With a membrane of A.P.D. 0.58μ the filtrate of the *pallidum*-containing suspension showed a fair number of spirochetes on microscopic examination, and a still larger membrane (0.74μ) could be used without the passage of ordinary bacteria. The treponemas, in spite of their considerable length (10 to 14μ) are able to traverse pores of relatively small diameter. Presumably their extreme slenderness and their active motility are factors in their ready filterability.

SUMMARY

Treponema pallidum, in suspensions of testicular tissue partly cleared by centrifugation, is able to pass through a collodion membrane of calculated pore diameter 0.4μ, as demonstrated microscopically and also by the infectivity of the filtrates. *Treponema novyi*, in rat plasma, readily passes through a membrane of 0.57μ A.P.D. but not one of 0.52μ.

The collodion membrane appears to offer the best method of separating spirochetes from accompanying bacteria.

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ATTEMPTS TO REVEAL SEX IN BACTERIA; WITH SOME LIGHT ON FERMENTATIVE VARIABILITY IN THE COLI-AEROGENES GROUP

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Received for publication October 2, 1936

More than ten years ago we carried out some extensive experiments on a problem which proved fruitless by our method of approach. It turned out to be a good idea that did not work. However, there were salvaged from those labors some data which we believe to be pertinent from the standpoint of variability in the coli-aerogenes group.

ORIGINAL OBJECT OF THE WORK

Although there would seem to be no point in even mentioning the useless part of the work, so many bacteriologists have told us it should be published that it will be briefly described.

We thought it possible that if conjugation or other primitive sexual processes occur in bacteria, this fact might be put in evidence by physiological tests. Not holding the most extreme views on bacterial variability, transmutability, etc., we decided to conduct the experiments only with very closely related organisms. For this purpose two strains of *Bacterium coli* (71 and 24) were selected, one (71) of which fermented salicin but not sucrose, while the other (24) fermented sucrose but not salicin. These cultures were inoculated into the same tube of 1 per cent peptone, the mixed culture incubated a week at 37°C., and plated at intervals for colony isolation. The reisolated daughter cultures were tested for ability to ferment sucrose and salicin. With the results of the first experiment in hand, others were initiated with added zeal until several thousand isolations had been made and tested on sucrose and salicin. If sexual

processes were involved one might expect to obtain, in addition to the two parent strains (sucrose +, salicin −) and (sucrose −, salicin +), a few strains which would be (sucrose +, salicin +) and others (sucrose −, salicin −).

The experiments were a grand success: but, unfortunately from the viewpoint of making sensational discoveries, the work was not discontinued soon enough. Besides the two parent types, from less than 1 to more than 10 per cent were obtained of (sucrose +, salicin +) and (sucrose −, salicin −) in the various experiments. We then did what intelligent investigators would probably have done in the beginning: the two pure parent strains were tested, and each yielded subcultures which were (sucrose +, salicin −), (sucrose −, salicin +), (sucrose +, salicin +), and (sucrose −, salicin −).

It is needless to state that our attempts to throw light on the possibility of sex in bacteria were unsuccessful; but, it should be noted, neither do they give evidence against the idea.

VARIATIONS IN FERMENTATION TESTS

It has just been shown that from each of two strains of *Bacterium coli* there were obtained substrains which were (sucrose +, salicin −), (sucrose −, salicin +), (sucrose +, salicin +), and (sucrose −, salicin −). In other words, from pure cultures of *Bacterium coli* there were obtained strains which might be considered as four different species according to some classifications of this group: *Escherichia coli*, *Escherichia vekanda*, *Escherichia communior*, and *Escherichia neapolitana*. Critics might contend that our isolations of (sucrose −, salicin −) cultures represented not variation but the mere existence of mixed strains in the parent cultures, e.g., (sucrose +, salicin −) and (sucrose −, salicin −). The work of Sherman and Albus (1937) shows the improbability of such a contingency with the use of properly prepared poured agar plates, but even granting that point, such a contention could not explain the (sucrose +, salicin +) strains. It should be added that our parent cultures were also recent colony isolations from stock cultures.

With our original object in view, these results were most dis-

appointing and further attempts were made to get more stable cultures and better tests. As we held salicin in rather low regard as a test substance, in spite of its great vogue among some students of the colon bacilli and the streptococci, it came under suspicion and was dropped. Tests were then run with a number of new cultures of *Bacterium coli* and *Bacterium aerogenes*, using mannitol in place of salicin. The results obtained with seven cultures are given in table 1.

An inspection of this table shows that, so far as the work went, two cultures of *Bacterium coli* (10 and 142) and one culture of *Bacterium aerogenes* (104) yielded only strains which agreed with

TABLE 1

Variations on sucrose and mannitol among substrains from pure cultures

PARENT CULTURE	NUMBER OF SUBSTRAINS TESTED	SUCROSE −; MANNITOL −	SUCROSE +, MANNITOL −	SUCROSE −; MANNITOL +	SUCROSE +; MANNITOL +
		per cent	*per cent*	*per cent*	*per cent*
Bact. coli (10)	100	0	0	100*	0
Bact. coli (142)	184	0	100*	0	0
Bact. aerogenes (104)	149	0	0	0	100*
Bact. aerogenes (88)	200	0	0	64*	36
Bact. aerogenes (54)	100	53*	30	10	7
Bact. aerogenes (106)	50	64*	30	4	2
Bact. aerogenes (37)	100	53	30*	10	7

* Type of parent culture.

the parent cultures in their respective actions on sucrose and mannitol. On the other hand, four cultures of *Bacterium aerogenes* (88, 54, 106, and 37) produced substrains which varied from the parent cultures.

Still bent upon getting stable strains for our "breeding" experiments, we attempted to eliminate variability by successive selection. The parent culture was plated; a daughter colony selected which gave fermentation reactions identical with those of the parent strain; this substrain in turn plated, and a typical daughter strain selected. This process was continued through four successive platings and substrain selections. At each plating about 100 colonies were selected and tested on sucrose and mannitol. The

data obtained on the first and fourth isolations with four cultures of *Bacterium aerogenes* are given in table 2.

It is seen that in two cases (54 and 106) marked progress was apparently made toward obtaining stable strains, in one case (88) no progress was made, while the fourth culture (37) wandered still further from its original base. None of the strains was stable after four selective isolations. In case any one could still worry about the possibility of mixed strains in the original cultures, we will only say that the chances against this being the case in the fourth isolations are more than six million to one (Sherman and Albus, 1937).

TABLE 2

Variations obtained in first and fourth successive colony isolations from four cultures of Bacterium aerogenes

CULTURE EMPLOYED	SUCROSE −; MANNITOL −	SUCROSE +; MANNITOL −	SUCROSE −; MANNITOL +	SUCROSE +; MANNITOL +
	per cent	*per cent*	*per cent*	*per cent*
54—First isolation	53*	30	10	7
54—Fourth isolation	93*	1	5	1
106—First isolation	64*	30	4	2
106—Fourth isolation	98*	0	2	0
88—First isolation	0	0	64*	36
88—Fourth isolation	0	0	64*	36
37—First isolation	53	30*	10	7
37—Fourth isolation	91	5*	4	0

* Type of parent culture.

One point should be especially emphasized: the original parent cultures were checked for their fermentative reactions after the completion of the work; all gave results identical with those found at the beginning. It would seem probable that individual cell variants commonly occur in stock cultures just as they do when isolated in agar. It is an exceedingly nice question, which we shall not now attempt to answer, why these variants do not ordinarily make their presence evident in mass cultures. It is believed, however, that the data herein reported throw light on those relatively rare but authentic instances in which stock cultures have changed in their fermentative capacities.

With the infallible judgment of hindsight, it is easily seen that such cultures as *Bacterium aerogenes*, strains 37, 54, 88, and 106, should have been maintained in stock cultures, and in selective media, for a long period of time, in an attempt to bring out variations in mass culture. But, with our original interests finally frustrated, the cultures were discarded in disgust. At this point, also, the pressure of other duties made necessary the discontinuance of this apparently futile study; futile, that is, from the viewpoint of its initial purpose.

Again reverting to our original objective, we can now only gaze wistfully upon strains 10 and 142 of *Bacterium coli*. These strains, obtained just before the conclusion of the work, were at least relatively stable and had just the right combinations of characteristics for our pet cross-breeding experiments. We have not entirely lost faith in the idea; and this tedious method of approach is commended to those so situated that they may impose upon willing and unsuspecting assistants.

DISCUSSION

The implications of our results on fermentative variation are of sufficient importance to merit the serious consideration of students of the coli-aerogenes group. By all means, they should be confirmed by others. We have no doubt of their confirmation by any one who will use a reasonably varied assortment of cultures and make a sufficient number of isolations from each. Our experience is too limited to be of much value, but will be stated for what it is worth: Our most stable strains were from old laboratory cultures, while those giving rise to the largest number of variants were from cultures recently isolated from natural sources.

These results were easily confirmed by Sherman and Hussong (1937) with an old laboratory culture of *Streptococcus cremoris*, but only with difficulty with a stock culture of *Streptococcus lactis*. On the other hand, Dr. L. R. Curtis, while a student in this laboratory, failed to get variant strains from a stock culture of *Streptococcus pyogenes*, although a very large number of isolations were made.

If these findings are confirmed by other workers, there would

appear to be nothing left of the fantastic classifications of the coli-aerogenes group based upon the fermentation reactions. When a rational classification is established for the true species which probably exist within the *Escherichia coli* group and the *Aerobacter aerogenes* group, it is fairly safe to predict that this much to be desired end will be accomplished by investigators who have the initiative to apply new and more incisive methods, and who are not too much bound by what has already been done. It is of more than passing interest that the foundation rock upon which rests the basic differentiation within the group as a whole—the gas ratio—has remained unused since the classical researches of Rogers and Clark (1914a, 1914b, 1915); and even Rogers' (1921) brilliant pioneer work on the aerogenes-proteus relationships has not been exploited by subsequent investigators.

In closing, notice should be taken of some points, perhaps of weakness, in the work here presented. One question that will arise in some minds is the matter of single-cell cultures. We do not think this matter is a valid, if indeed even a pertinent, objection to the methods used. Our exploratory tests (Sherman and Albus, 1937) support the essential integrity of the procedure followed. Another and more pertinent question concerns the colonial types of the substrains which varied in fermentative properties from the parent cultures. We have no information on this point: Our original interest was not in variation in the usual sense and not at all in the more restricted sense of dissociation. An important point is whether the variations with which we dealt were indeed qualitative ones and not gross quantitative differences. We believe them to be qualitative. The fermentation tests were made in a medium containing only 1 per cent peptone with the addition of the test substance, and the presence of fermentation was judged by changes in pH, determined colorimetrically. In such a lightly buffered medium, slight fermentation causes rather emphatic changes in pH, and as a matter of fact we were not troubled by feeble or borderline reactions in this work.

Finally, we are perhaps obligated to say a word concerning the nature of the phenomena reported. It is our view that they

should be considered simply as cellular mutations. In the present state of our knowledge, it is probably wise to leave aside those genetic concepts and terms which have nuclear or sexual implications.

SUMMARY

From pure cultures of *Bacterium coli* and *Bacterium aerogenes* substrains were obtained which varied from the parent strain in the fermentation of test substances.

For example, from a culture of *Bacterium coli* which fermented salicin but not sucrose, substrains were obtained which were (sucrose −, salicin −), (sucrose −, salicin +), (sucrose +, salicin −), and (sucrose +, salicin +). Likewise, a culture of *Bacterium aerogenes* which fermented neither sucrose nor mannitol yielded substrains which were (sucrose −, mannitol −), (sucrose +, mannitol −), (sucrose −, mannitol +), and (sucrose +, mannitol +).

Some cultures yielded only substrains which were true to the parent type, in so far as the work went.

Efforts to reveal sex in bacteria by the methods employed proved futile.

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THE WATER CONTENT OF BACTERIAL SPORES

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Received for publication October 8, 1936

The explanation most usually offered for the resistance of bacterial spores to temperatures which will destroy the vegetative cells of the same species is based on the fact that the water content of protein materials determines the coagulation temperature. From a survey of the literature on this subject it would appear that the statements made by the authors of bacteriological texts that bacterial spores contain from 10 to 15 per cent water are based on speculation. It is true that such a difference in the water content of vegetative and spore forms of a bacterial species would help to explain the proven difference in heat resistance between these two types of cells. It is equally true, however, that the only record, which we have been able to discover, of an actual determination of the water content of bacterial spores made prior to 1933 (Dyrmont (1886)) reports 85 per cent water in the spores of *Bacillus anthracis*. More recent work by Virtanen and Pulkki (1933) using *Bacillus mycoides*, has shown that there is no significant difference in the water content of the two types of cells.

The determination of the water content of microörganisms presents two major difficulties not encountered in similar investigations with larger forms: (1) Special methods must be used in order that the cells be predominately vegetative or spore forms, and (2) some base or point of reference must be established. The first of these two can be rather readily overcome by the choice of medium and proper conditions. Virtanen and Pulkki made a rather elaborate study of the factors influencing the formation of spores and developed media suitable for obtaining

vegetative forms of *Bacillus mycoides* with few or no spores, and spore suspensions with very few vegetative cells. The media and methods used in the work reported below were those of the above-mentioned authors modified in minor details.

The organisms used were from stock cultures, maintained over a period of years, of *Bacillus subtilis*, *Bacillus mycoides* and *Bacillus megatherium*. A basic medium of the following composition was used; the vegetative cells were harvested from broth, whereas 1.75 per cent agar was added to the medium for spore production.

Glucose	2.5 grams
Peptone	5.0 grams
Sodium chloride	0.5 gram
Dipotassium phosphate	5.0 grams
Ammonium sulphate	2.5 grams
Water, distilled	1000 ml.

The medium was adjusted to pH 6.9 to 7.3 and autoclaved. After sterilization a slight crystalline precipitate occasionally appeared. This precipitate was soluble in distilled water and hence did not interfere with the final determinations.

Spores were obtained by growing the organisms on agar in flat bottles for 30 to 45 days at room temperature. They were removed from the substrate by gentle washing and strained through gauze to remove adhering agar. The spores were washed with distilled water four times, dried between filter paper, and transferred to a 100 cc. platinum dish for weighing. Microscopic examination was made to determine the relative numbers of vegetative and spore forms and detect any debris which might be present; also a petrographic microscope was used to determine whether or not all salts had been removed.

The vegetative cells were grown in 500 cc. flasks containing 180 cc. of medium at 37°C. After preliminary concentration by centrifugation the cells were washed 4 times in distilled water, drained and treated in the same manner as were the spores. No more than 32 hours elapsed from the time of inoculation of the medium until the vegetative mass was in the platinum dish for weighing. About 30 Erlenmeyer flasks were inoculated for each determination.

The second difficulty referred to above, was to establish a base or point of reference. With the method used it is impossible to determine when the surfaces of microörganisms are free of water, while the total water content of the cells is still undisturbed. However, by identical treatment it was hoped that blotting would remove an equal amount of the water adhering to the surface of the two types of cells and give a satisfactory working basis. The difference in the size of the spores and vegetative cells for a given species will, however, cause a considerable error at this point. Air-dry weights as points of reference are similarly open to criticism because of the possibility of unequal permeability of the cell walls in the two forms, resulting in a greater loss through evaporation in one case than in the other.

It was hoped that by weight determinations at several points during the drying process large errors due to such conditions as those mentioned would be detected if they occurred. The uniform and consistent loss in weight by each method of drying and the similarity in the results obtained in the three species used leads us to believe that errors due to a lack of a suitable base are not of great importance. All determinations for the two types of cells for a given species were carried on concurrently so that conditions were identical.

After blotting the cell masses for 30 minutes the initial weights were determined. These are termed moist weights. Air drying was accomplished at temperatures varying from 18.5° to 20.5°C. and was continued for 5 to 6 days until a constant weight was reached for any given atmospheric condition. The relative humidity varied from 70 to 80 per cent. The third weights were obtained after desiccation to constant weight over fresh calcium chloride at atmospheric pressure. The desiccation was followed by drying in an oven at 105° to 110°C. for approximately 4 days. The ashing was accomplished by the usual method in platinum crucibles.

Table 1 gives the weight in grams of the spore and vegetative forms of the three species at the various stages in the drying process.

However, a clearer idea of the amount of water lost at each point in the progressive drying may be had if each of the three

stages is successively used as a base and the weight of the cells at that point is given a value of 100. By this means the percentage loss of water may be determined for each stage.

TABLE 1

ORGANISM	TYPE OF CELL	MOIST WEIGHT*	AIR DRY WEIGHT	DESICCATED WEIGHT	OVEN DRY WEIGHT	ASH WEIGHT
B. subtilis...........	100% vegetative		0 9926	0.8966	0.8189	0.0492
	97% spore		0 8882	0 7906	0.7483	0.0593
	98% spore		0.4874	0.4369	0.4167	0.0387
B. megatherium.	100% vegetative	5.2067	1.2346	1.1220	1.0573	0.0500
	100% spore	1.5157	0.7452	0.6759	0.6323	0.0491
B. mycoides.........	100% vegetative	3.7172	0.5096	0.4753	0.4534	0 0298
	100% spore	0.9871	0.3339	0.3061	0.2901	0.0356

* All weights in grams.

TABLE 2

ORGANISM	TYPES OF CELL	MOIST WEIGHT = 100					AIR DRY WEIGHT = 100					DESICCATED WEIGHT = 100				
		Moist weight	Air dry weight	Desiccated weight	Oven dry weight	Ash weight	Moist weight	Air dry weight	Desiccated weight	Oven dry weight	Ash weight	Moist weight	Air dry weight	Desiccated weight	Oven dry weight	Ash weight
B. subtilis . .	100% vegetative							100	90 3	82.5	5 0		110.7	100	91.3	5.5
	97% spores							100	89 0	84 2	6 7		112 2	100	94.6	7 5
	98% spores							100	89.6	85 5	7.9		111.6	100	95.4	8.9
B. megatherium .	100% vegetative	100	23 7	21 6	20 3	0.96	419 5	100	90 0	85 6	4 0		110 0	100	94 2	4.6
	100% spores	100	48 9	44.6	41 8	3.2	203 5	100	90 7	84 8	6 6		110 3	100	93.5	7.3
B. mycoides . ..	100% vegetative	100	13.7	12 8	12 2	0.82	730.0	100	93.3	89.0	5.8		107 2	100	95.4	6.3
	100% spores	100	33 8	31 0	29 4	3.6	298 0	100	91.7	86.9	10.7		109.1	100	94.8	11.6

From the figures in table 2 it will be seen that if the moist weight obtained by weighing blotted cells, is considered to represent the total cell content, a definite difference in water content of the vegetative and spore forms is found. Assuming that oven

drying removed all of the water from both types of cells, the water content of the vegetative cells of *Bacillus megatherium* would be calculated to be 79.7 per cent while that of the spores would be 58.2 per cent on a moist basis. The vegetative forms of *Bacillus mycoides* would show 87.8 per cent water and the spores 70.6 per cent.

In considering the percentages based on moist weights it must be remembered that there is no analogy between the blotting of a mass of bacterial cells and the drying of the surface of larger forms. In the latter case most if not all surface water is removed and any which remains is of negligible importance because the surface is small as compared to the total mass. The surface is relatively large in the case of bacterial cells and occluded water is difficult to remove. Nor is it possible to assume that the amount of water removed from two dissimilar masses of bacterial cells by blotting would be the same. On the contrary, it would seem highly probable that the tangled mat of vegetative cells of either *B. mycoides* or *B. megatherium* would retain more water than would a group of discrete spores.

If the air-dry weight of the bacterial cells is chosen as representing 100 per cent of the cell contents, the water lost by oven drying of the vegetative cells of *B. subtilis* is found to be 17.5 per cent while the losses in the two samples of spores used were 15.8 and 14.5 per cent. *B. megatherium* showed a loss of 14.4 per cent for the vegetative cells and 15.2 per cent loss for the spores. The vegetative forms of *B. mycoides* lost 11 per cent whereas the spores lost 13.1 per cent.

Using the air-dry weight as the starting point for a comparison of the water content of the two types of cells is, of course, open to one serious criticism: namely, that a difference in the permeability of the walls of the two forms is quite probable. If the spore wall is less permeable to the passage of water than is the cell wall of the vegetative form a greater loss would occur in the latter type during the air drying process and would result in a low water loss by subsequent oven drying.

However, such differences in the permeability of these nonliving structures would be expected to persist. If the percentage

of water lost from the two types of cells between air drying and calcium chloride desiccation or between air drying and oven drying are compared, it will be found that the loss is very similar for the spore and vegetative forms in each of the three species. The calculations based on the weight of the cells after desiccation with calcium chloride serve only to emphasize the uniform loss of water from both cell types in the case of *B. megatherium* and *B. mycoides*. There is, however, a difference in the amount of water lost by the vegetative and spore forms of *B. subtilis*.

From the consideration of the results obtained by Virtanen and Pulkki and of the data presented above, which are in very close agreement with their work, the following conclusions seem justified:

1. The use of the moist weight of a mass of bacterial cells as a point of reference in the determining of the amount of water within the cells is open to objection. Likewise the use of this method to compare the moisture content of two masses of dissimilar structure may give rise to gross errors.

2. The difference in water content of vegetative and spore forms of at least three species of bacteria is not sufficiently great to explain the remarkable thermostability exhibited by the spores, regardless of which weight is chosen as the basic one.

While the above data seem to rule out the possibility of differences in water content being responsible for the difference in heat resistance between the two types of cells they offer no basis for any other explanation. Virtanen and Pulkki suggest the possibility that the enzymes responsible for growth may be in a different state in the two types. The evidence which they present tends to indicate that the resistance is intimately tied up with enzyme survival but furnishes no proof of a difference in enzymes. Another explanation of this phenomenon lies in the possibility that bound water may be inactive, in so far as its influence on the coagulation of protein material by heat is concerned, in a manner analogous to its action in preventing the death of plants by freezing or by drought as shown by the work of Newton and Martin (1930).

It is conceivable that a sufficiently high percentage of the

water in a bacterial spore might be bound, and reactions which would be comparable to those one would expect if the cell contained the small quantity of water with which it is usually credited might result, although the gross water contents of the two types of cells might be very nearly the same.

Bound water determinations are being made upon vegetative and spore forms of bacteria in an attempt to determine whether or not this factor is of importance in the heat resistance of spores.

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OPTICAL ACTIVITY OF LACTIC ACID PRODUCED BY L. ACIDOPHILUS AND L. BULGARICUS

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Received for publication October 10, 1936

Experiments dealing with the isomeric forms of lactic acid produced by *Lactobacillus acidophilus* and *Lactobacillus bulgaricus* have failed to take into account the possibility that R and S dissociants might play a determining rôle. The results reported show wide discrepancies varying from active to racemic lactic acid, including mixtures of both (Bertrand and Weisweiller, 1906; Heinemann and Hefferan, 1909; White and Avery, 1910; Currie, 1911; Kopeloff and Bass, 1927; Hyde and Hammer, 1927; and Curran, Rogers and Whittier, 1933).

In the present study laboratory cultures were used which were originally obtained from the sources noted below and for which grateful acknowledgment is made.

NUMBER	LACTOBACILLUS	FORM	SOURCE
K	*acidophilus*	R	N. Kopeloff
1482-A	*acidophilus*	S	H. K. Mulford Company
B-4-U. S.	*bulgaricus*	R	W. R. Albus
B-12-U. S.	*bulgaricus*	S	W. R. Albus

The identity of the K and 1482A strains of *L. acidophilus* has been verified by repeated passage and recovery from the human intestinal tract. The failure to implant B-4-U. S. was reported by us (Kopeloff, 1926) and Kulp (1926) and we have also failed to implant B-12-U. S. in the human intestinal tract. The acidophilus cultures mentioned have been further differentiated from the bulgaricus cultures by the surface tension test (Kopeloff and Beerman, 1927).

Milk cultures of each of the above pure strains were incubated for $3\frac{1}{2}$ weeks at 35°C. The chemical methods employed were those described by Pederson, Peterson and Fred (1926), omitting

TABLE 1

Zinc lactates from L. acidophilus and L. bulgaricus

CULTURE	FRACTION	WEIGHT	WATER OF CRYSTALLIZATION	PER CENT SOLUTION	$[\alpha]_D^{27°}$
			L. acidophilus		
		grams	*per cent*		
K rough	1	11.3	18.2	1 03	0
	2	4.2	18 2	1.02	0
	3	4.0	18.4	1 03	0
	4	4.0	18 3	1.08	0
	5	4.3	18.3	1.03	0
	6	3.0	18 4	1 02	0
	7	2 4	18 3	1.03	0
1482-A smooth	1	4 0	12 9	0 84	−8 85
	2	2 9	12 9	1.25	−8 36
	3	1 8	12 9	0 97	−7.74
	4	3.9	12 7	0 95	−9 00
	5	2 5	13 3	1 27	−8.22
	6	4 7	13 0	1.15	−8 37
			L. bulgaricus		
B-4-U. S. rough	1	2.7	18 2	1.00	0
	2	5.2	18 2	1 00	0
	3	4.2	18 4	1 00	0
	4	2.1	18 3	1 00	0
	5	0.7		1 00	0
	6	0 8	18 1	0 57	0
	7	3.3	12 6	1.46	−6 63
B-12-U. S. smooth	1	7 8	13.0	1 05	−8 58
	2	6.1	12.9	1 00	−8 82
	3	2.9	13.1	0.85	−7.82
	4	3.1	13.2	1 01	−7.47

sulfuric acid. The zinc salts were polarized in about 1.0 per cent solutions after purification and recrystallization.

The R form of *L. acidophilus* yielded inactive lactic acid. The zinc salts obtained contained 3 molecules of water of crystalli-

zation, the 7 fractions varying from 18.2 to 18.4 per cent (theoretical 18.2).

The S form of *L. acidophilus*, however, produced lactic acid, the zinc salt of which on fractionation gave polarimetric readings from $[\alpha]_D^{27°}$ −7.74 to −9.00 in the 6 fractions. Two molecules of water of crystallization were present, the results ranging from 12.7 to 13.3 per cent (theoretical 12.9). Thus the S form produced only dextro-rotatory lactic acid.

Similarly the R form of *L. bulgaricus* was inactive in the first 6 fractions with water of crystallization varying from 18.1 to 18.4 per cent. A seventh fraction gave a polarimetric reading of $[\alpha]_D^{27°}$ −6.63 with 12.6 per cent water of crystallization. This fraction represents only one-sixth of the total amount of zinc salt.

The S form of *L. bulgaricus* yielded polarimetric readings in 4 fractions from −7.47 to −8.82 with 2 molecules of water of crystallization present in the zinc salts, varying from 12.9 to 13.2 per cent. Dextro-rotatory lactic acid alone was therefore produced by a pure strain of *L. bulgaricus*.

Since these results seem to indicate a relationship between dissociants and the optical activity of the lactic acid produced, it is not unlikely that some of the inconsistencies reported by previous investigators might be explained on this basis. Since so few strains were used our results should be considered suggestive rather than conclusive. However it is tempting to correlate them with the findings of Upton and Kopeloff (1932) who used the same strains. They showed that the rough form appeared to be antigenically distinct from the smooth form in both *L. acidophilus* and *L. bulgaricus*. Further, the rough forms as well as the smooth forms of the two species were serologically indistinguishable.

SUMMARY

Using a single strain of each culture:

1. The R form of *Lactobacillus acidophilus* produced inactive lactic acid. The R form of *Lactobacillus bulgaricus* produced inactive lactic acid in the first 6 fractions, while the seventh yielded the dextro-rotatory enantiomorph. The latter represented one-sixth of the total zinc salt.

2. The S forms of both *L. acidophilus* and *L. bulgaricus* produced dextro-rotatory lactic acid.

3. It is suggested that some of the inconsistencies reported in the literature might be due to the use of cultures containing mixtures of R, S, or intermediate forms.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

EASTERN PENNSYLVANIA CHAPTER

PHILADELPHIA COUNTY MEDICAL SOCIETY BUILDING, JANUARY 26, 1937

ESSENTIAL IMMUNIZING ANTIGEN OF PNEUMOCOCCI. *Lloyd D. Felton*, The Johns Hopkins University, Department of Pathology and Bacteriology, Baltimore, Md.

The essential immunizing antigen of the pneumococcus was defined as that fraction of the cell which contained as many as, or more, immunizing doses, than the original cell from which the fraction was derived. Ten grams of Type I pneumococci dried from acetone were separated into five successive fractions: (A) acetone-soluble constituent of the cell; (B) HCl-insoluble at pH 3; (C) HCl-soluble, insoluble with 2 volumes of alcohol; (D) acid-alcohol-soluble, insoluble on neutralization; and (E) alcohol-neutral-soluble fraction. (A) showed no active immunizing value for white mice, a confirmation of Wadsworth's work. (B) contained active immunizing antigen, but because it was composed of many undissolved cells, this fraction was eliminated from consideration. (C) when dried (0.607 gram) immunized mice in dilution of 1:50,000,000, had a precipitin titer of 1:5,000,000, contained 12 per cent hydrolyzable sugar, and 8.3 per cent nitrogen. (D) gave a precipitate (0.439 gram) with immunizing titer of 1:50,000 and precipitin titer of 1:80,000. (E) on evaporation gave a slightly positive Molisch reaction, no precipitinogens, and failed to immunize white mice.

The original cells (10 grams) immunized mice in 1:1,000,000 dilution (0.5 cc. dose). Therefore, there was a total of 10,000,000 immunizing units. The acid-alcohol-insoluble fraction (0.607 gram) immunized mice in dilution 1:50,000,000 and thus contained 30,000,000 immunizing doses (0.607 × 50,000,000). Hence, disregarding the slight immunizing activity of the other fractions, there were found at least three times as many immunizing doses as in the original cells. This fraction, polysaccharide in nature, was not a pure substance.

Another 23 grams of 16-hour growth, fractionated in the same way, yielded 2.21 grams of this same C fraction. The total number of units in the cells was 11,500,000 and in the C fraction 110,000,000. The 48-hour growth (17 grams) contained 8500 immunizing doses, and the 0.190 gram isolated contained 1,900,000 immunizing doses. These experiments were conducted with Type I organisms. It has been found repeatedly that Type II cells act in the same manner.

This fraction produces specific immunity in mice, and heterologous immunity in human beings. However, without purification it may give very severe reactions in human beings.

NEW YORK CITY BRANCH

TEACHERS COLLEGE, COLUMBIA UNIVERSITY, FEBRUARY 9, 1937

THE DIFFERENTIATION AND DIAGNOSTIC CHARACTERISTICS OF FOUR FUNGI CAUSING SYSTEMIC INFECTIONS. *Rhoda W. Benham.*

Under the name blastomycosis have been grouped a number of the deep-seated fungus infections. This has led to a great deal of confusion as many of these conditions are quite distinct both clinically and etiologically. This can be shown by study of the fungi concerned.

1. American blastomycosis: In this condition the causative organism *Blastomyces dermatitidis* appears in the lesion or in pus from the lesion as spherical budding cells with refractile walls. In culture a white, filamentous growth forms, with large round or pear-shaped spores borne singly on the hyphae, either sessile or on short side branches.

2. Cryptococcosis (European blastomycosis or torula infection): In this disease the organism *Cryptococcus hominis* appears in the lesion as budding cells with wide gelatinous capsules. In culture, budding cells likewise surrounded by a capsule are found. The culture is of a moist, mucoid consistency.

3. Coccidiodal granuloma: The organism *Coccidiodes imitis* forms in tissue large spherical, thick-walled cells which reproduce by endospores. There is no budding. In culture a white mycelial growth forms. There are no spores other than chlamydospores.

4. Meningeal moniliasis: The organism, *Monilia albicans* forms in the lesion budding cells, and elongated forms suggesting mycelia. A pasty yeast-like growth occurs. On suitable media the characteristic mycelium with clusters of budding cells and chlamydospores is found.

Each of these conditions can be correctly diagnosed by the characteristic picture of the organism in the diseased tissue. In the case of "1" and "3" animal inoculation is the most reliable means of diagnosis as the cultures are easily confused.

RECENT EXPERIMENTS ON ANTI-RABIC VACCINATION. *L. T. Webster,* Rockefeller Institute, New York City.

THE INTRACELLULAR CRYSTALLIZATION OF STANLEY'S TOBACCO VIRUS PROTEINS. *H. P. Beale,* of Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York.

A microscopic examination of plants systematically infected with ordinary tobacco mosaic virus discloses the presence of large crystalline substances deposited chiefly on the external layers of host tissue. The crystalline material is confined to the chlorotic areas of the leaf and is not present in the green areas of diseased leaves nor in healthy leaves. The crystalline inclusions appear to be specific for tobacco mosaic disease and occur as plates, definitely hexagonal at times, in side view oblong.

Pieces of epidermal tissue are stripped off the back of the midrib of diseased leaves and mounted in water. A few drops of a saturated solution of magnesium sulphate or dilute acid, such as hydrochloric, sulphuric, acetic, or nitric, are run under the cover slip and the crystalline material is gradually transformed into a mass of needle crystals, bearing a striking resemblance to Stanley's needle crystals,

obtained by a similar salting out or acidification of purified, concentrated virus extract. The intracellular crystals are denatured by an acidity greater than about pH 1 or an alkalinity in excess of about pH 11.8. Recrystallization can not be induced by readjusting the reaction of the host cell to neutral. Stanley's crystalline tobacco protein becomes denatured when subjected to the same limits of H-ion concentration (Science, **81**: 644–645. 1935).

It is concluded from these observations that the intracellular crystalline deposits associated with tobacco mosaic disease are the source of Stanley's crystalline proteins obtained from tobacco virus extract.

DECOMPOSITION OF CARBOHYDRATES AND ALCOHOLS WITH PRODUCTION OF GAS AT 46°C. BY MEMBERS OF THE GENUS ESCHERICHIA

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Received for publication May 18, 1936

Although it has been known for many years that *Escherichia coli* grows rapidly and luxuriantly at a temperature well above that of the body, only a few workers (Leiter, 1929; Williams, Weaver, and Scherago 1933; Perry and Hajna, 1933 and 1935; Hajna and Perry, 1935) have utilized higher temperatures either in the isolation of this organism or in the study of its fermentation of various carbohydrates or alcohols. As far as the author is aware, only glucose (Eijkman, 1904; Perry and Hajna, 1933, Hajna and Perry, 1935; Skinner and Brown, 1934), mannitol (Bulir, 1907; Minkewitsch, 1929; Skinner and Brown, 1934), and lactose (Levine, Epstein and Vaughn, 1934) have been utilized in the investigation of the ability of *Escherichia coli* to ferment at, or around, the temperature of 46°C.

The following investigation was therefore undertaken to determine whether the members of the genus *Escherichia* can decompose carbohydrates and alcohols other than those referred to, at this temperature.

METHODS AND MATERIALS

Cultures. Cultures isolated from human feces, were grouped on the basis of (1) fermentation of sucrose, sorbitol, dulcitol, adonitol, and salicin at 37°C.; and (2) the degree of roughness and smoothness as indicated by their colony characteristics on eosin methylene-blue agar plates. (See table 1.)

Inoculation of cultures. Inoculations were made at first by a

loop from 24-hour broth cultures and subsequently (as a check method) by a straight needle.

Temperature and duration of incubation. Duplicate tubes of the media were inoculated at room temperature. One series of tubes was incubated at 46°C. while the other series was incubated at 37°C. Wire test-tube racks were used to permit free circulation of air about the tubes with the result that the temperature

TABLE 1

Cultural characteristics of strains

Total strains 129

REACTIONS AND TESTS	NUMBER OF STRAINS USED											
	18	15	2	17	3	3	6	4	7	11	25	18
	A	B	C	D	E	F	G	H	I	J	K	L
Sucrose	—	—	—	—	—	—	—	AG	AG	AG	AG	AG
Sorbitol	—	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG
Dulcitol	—	—	AG	AG	—	—	—	—	—	AG	AG	AG
Adonitol	AG	AG	—	—	—	—	—	—	—	—	—	—
Salicin	—	—	—	AG	AG	AG	—	—	AG	—	AG	AG
Type of colony on E.M.B. plate	S	S	S	S	S	R	S	S	S	S	S	R

Sugars: — = no reaction in 72 hours; AG = acid and gas, at 37°C.

Plate: S = smooth phase; R = rough phase.

All of the 129 strains ferment glucose, levulose, d-mannose, d-galactose, xylose, rhamnose, lactose, maltose, trehalose, and d-mannitol with the production of acid and gas; do not utilize citrate as source of carbon; produce indol; do not hydrolyze hippurate;* are methyl-red positive; do not produce acetylmethylcarbinol.

Composition of carbohydrate broth: Meat-extract, 0.3 per cent; peptone, 1 per cent; NaCl, 0.5 per cent; carbohydrate, 0.5 per cent; brom thymol blue indicator (1.6 per cent alcohol solution), 0.4 cc. per 100 cc. of medium.

* Synthetic medium of Hajna and Damon, Amer. Jour. Hyg., 1934, **19**, 545.

of the medium in the tubes reached 46°C. in approximately an hour.

Gas production in the media was noted at 24, 48 and 72 hours. The final readings as given in the tables are those made at 72 hours.

Type of incubator used. A Castle precision incubator as described in a previous paper (Perry and Hajna, 1933) was used

throughout the investigation. The thermoregulator was of the capsule type. The temperature fluctuation from 46°C. was at most ±0.5°.

A tube temperature of 46°C. was selected for use in this study basing on the results obtained by Perry and Hajna[1] in the recent (unpublished) study of influence of various factors in the Eijkman test. The tube temperatures, used in that study, ranged from 43° to 49°C. It was found that, in a favorable medium, bacteria of the genus *Escherichia* (human origin) outgrow other members of the colon group. *Citrobacter* organisms, *Aerobacter cloacae*, and *Aerobacter aerogenes* (of water origin) failed to produce gas from glucose at 46°C. whereas all of the organisms of the *Escherichia* group and a few of the *Aerobacter aerogenes* (of human origin) produced gas.

TABLE 2

Composition of various basic media

INGREDIENTS	MEDIUM I	MEDIUM II	MEDIUM III	MEDIUM IV†	MEDIUM V	MEDIUM VI	MEDIUM VII	MEDIUM VIII
	grams per liter	*grams per liter*	*grams per liter*	*grams per liter*	*grams per liter*	*grams per liter*	*grams per liter*	*grams per liter*
Peptone (Bacto)	10	10	10	15	10	10	10	10
NaCl	5	5	5	5	5	5	5	5
Beef extract (Bacto)	–	3	3	–	–	–	–	–
K_2HPO_4	–	–	4	4	–	4	–	4
KH_2PO_4	–	–	1 5	1 5	–	1 5	–	1 5
Sugar-freed beef infusion*	–	–	–	–	1 liter	1 liter	–	–
Beef infusion (not sugar-freed)	–	–	–	–	–	–	1 liter	1 liter

* After the method of Skinner and Brown (1934).

† Basic medium of Perry and Hajna (1933 and 1935).

Note: Final pH of all media 7.0.

Basic media used. It is well known that the amount and type of protein and the buffering qualities of the media employed influence the amounts of acid and gas produced from carbohydrates. Various brands of peptone (Bacto-peptone, Bacto-neopeptone, Bacto-proteose peptone, and Bacto-tryptone) were tested by Perry and Hajna for use in the Eijkman test. Bacto-

[1] Unpublished work, 1936, "Further Studies on the Eijkman Test."

peptone was eventually selected for use in this study as consistent results were obtained by the use of this brand of peptone.

The concentration of buffer, as stated in table 2, was likewise determined beforehand.

Eight media varying in regard to these factors were, therefore, used. (See table 2.) No acidity was observed in any of the sterilized media except that containing fructose.

EXPERIMENTAL RESULTS

All of the strains described grew abundantly in all eight basic media containing the fifteen carbohydrates and alcohols, at both 37° and 46°C. Fermentation was decided solely on the basis of gas production. (See table 3.)

MONOSACCHARIDES

Xylose and arabinose. Xylose and arabinose were easily decomposed with evolution of a moderate amount of gas in all of the nutrient media at 46°C. However, a larger volume of gas was evolved in media with buffers and in non-sugar-free beef infusion. Observations at 37°C. were identical with those at 46°C. with both of these sugars and in all media.

Rhamnose. Although rhamnose was decomposed, the amount of gas was small both at 37° and at 46°C. in all media.

Glucose, mannose and fructose. A considerable amount of gas was produced by all strains from d-glucose, d-mannose and fructose and did not vary widely in any of the media employed. More gas was, however, produced in buffered media than in unbuffered, and in beef infusion not sugar-free than in sugar-free beef infusion.

Galactose. D-galactose was decomposed with difficulty at 46°C. if the medium contained beef extract and no buffers or if the medium contained beef infusion free of muscle sugar. In buffered peptone broth, the reactions were identical with those of d-mannose at 46°C.

DISACCHARIDES

Trehalose. Trehalose is generally thought to be more resistant to hydrolysis than sucrose. Contrary to expectation, trehalose

TABLE 3

Production of gas from carbohydrates and alcohols at 37° and 46°C. by members of the genus Escherichia

CARBOHYDRATE OR ALCOHOL	MEDIUM	CULTURES																							
		A		B		C		D		E		F		G		H		I		J		K		L	
		37°	46°	37°	46°	37°	46°	37°	46°	37°	46°	37°	46°	37°	46°	37°	46°	37°	46°	37°	46°	37°	46°	37°	46°
Sucrose	All	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	+	−	+	−	+	−
Maltose	I	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−
	II, V	+	v	+	v	+	v	+	v	+	v	+	v	+	v	+	v	+	v	+	v	+	v	+	v
	Rest of media	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Trehalose	I, II, III	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v
	Rest of media	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dulcitol	All	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	+	−
Adonitol	All	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Sorbitol	I, II	−	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−
	Rest of media	−	−	+	v	+	v	+	v	+	v	+	v	+	v	+	v	+	v	+	v	+	v	+	v
*Rest of carbohydrates	All	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

\+ = gas produced; − = gas not produced; v = variable gas production.

* All of the strains fermented arabinose, xylose, rhamnose, glucose, mannose, fructose, lactose, galactose, and mannitol with the production of gas both at 37° and 46°C.

was readily decomposed in buffered media by the bacteria of the genus *Escherichia* at 46°C. In unbuffered beef extract broth or in sugar-free beef infusion, it was slowly decomposed. From 30 to 70 per cent of gas was produced in buffered media and in beef infusion medium not sugar-free.

Sucrose. The sucrose-fermenting strains decomposed sucrose readily at 37°C. in all the basic media. However, at 46°C. only one group (H) fermented sucrose. Gas occurred in all media both at 37° and 46°C.

Maltose and lactose. Maltose and lactose offer contrasting pictures. At 46°C. all strains failed to produce gas from maltose in plain peptone broth; they were able to attack this sugar slightly when beef extract or sugar-free infusion was added to the peptone and salt (media II and V). A small amount of gas was formed when buffers were added to a peptone-beef-extract medium or when beef infusion (not sugar-free) was added to peptone broth (media III and VII). In the three remaining media (IV, VI and VIII), maltose was decomposed slowly but certainly with the evolution of a large amount of gas in 72 hours. These media were well-buffered.

Lactose was fairly well decomposed with the production of gas in 24 hours in a medium containing only peptone and sodium chloride. The addition of beef extract or sugar-free beef infusion did not result in a material increase in gas production from this sugar but with the addition of buffers, more gas was evolved. The same reaction was observed in beef infusion not freed of muscle sugar without added buffers.

At the temperature of 37°C., both carbohydrates were readily decomposed.

ALCOHOLS

Although d-mannitol, d-sorbitol, and d-dulcitol are stereoisomers, d-mannitol, the alcohol of d-mannose, is more readily decomposed than d-sorbitol, the alcohol of d-glucose. The alcohols offer, in the order given, increasing difficulties to microbic decomposition with gas production.

Mannitol. In all of the media employed, d-mannitol was

regularly attacked by all the strains studied. Maximum gas production occurred, however, in buffered media. The average production of gas in 24 hours was 50 to 90 per cent in the inner tube, both at 37° and 46°C.

Sorbitol. Variable results were obtained with d-sorbitol at 46°C. The sorbitol-fermenting types produced more gas from sorbitol in buffered media.

Dulcitol and adonitol. In no instance was gas production from dulcitol or adonitol obtained at 46°C., although at 37°C. the dulcitol-fermenters and the adonitol-fermenters were able to produce gas respectively from dulcitol and adonitol in all of the media without difficulty. These two alcohols, however, were best decomposed with gas in buffered media.

INFLUENCE OF PROTEIN COMPOSITION ON GAS PRODUCTION

Although Eijkman (1904) used plain peptone broth plus glucose in his work, in which he distinguished the coli-form strains of warm-blooded animal origin from those of cold-blooded animal origin, this broth is the poorest culture fluid medium for production of gas from carbohydrates by bacteria of the genus *Escherichia* at 46°C.

Perry and Hajna and de Graaf (1928) have demonstrated that different results might be obtained when various brands of peptone are utilized in the Eijkman test. This observation has to be borne in mind when interpreting results and formulating conclusions as to the value of the temperature of 46°C. in the isolation and study of bacteria of the genus *Escherichia.* In this study, Bacto-peptone was used throughout.

The presence of meat extract in media seems to interfere with the production of gas although growth was heavy at both 37° and 46°C.

In beef-infusion broth, free of muscle sugar and with the carbohydrates under investigation added, many of the *Escherichia* strains produced only a small amount of gas. With the addition of buffers to the infusion broth, gas production was equivalent to that in media made from unfermented broth. The addition of buffers to the unfermented broth, however, made it superior

to that of the buffered sugar free infusion broth. This observation is in agreement with that of Brown (1921). It is well known that beef infusion broths vary greatly in their carbohydrate content and other growth stimulating factors, and those broths that are freed from muscle sugar are comparatively less nutritive than untreated broths.

BUFFERING EFFECTS

Isolation of cultures from unbuffered media was almost impossible in contrast to the ease of isolation from the buffered media even after 72 hours of continuous incubation at 46°C. The presence of buffer in the medium aids in holding the concentration of hydrogen-ions below the toxic limit of acidity, thus permitting a larger amount of sugar or alcohol to be decomposed with a larger amount of gas evolved. This confirms another observation made by Brown in regard to the value of buffers in media.

SUMMARY

A study has been made of the action of bacteria of the genus *Escherichia* on seven monosaccharides, four disaccharides, and four alcohols at 37° and 46°C. Eight different basic media were used in order to observe the effect of buffers, peptone, meat infusion, meat extract on gas production. In all, 129 strains were studied.

Buffers were found to enhance gas production at both 37° and 46°C. In a well-buffered basic medium, all the sugars and alcohols tested, except dulcitol and adonitol which were never fermented with gas at 46°C., were readily decomposed at both temperatures. In an adequately buffered medium, all the bacteria used remained viable after an incubation period of 72 hours.

Gas production is likewise influenced greatly by the type of protein in the medium. Meat extract was found to restrain gas production. Gas production tended to be less in meat infusion freed of muscle sugar.

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BACTERIAL VARIATION AS STUDIED IN CERTAIN UNSTABLE VARIANTS[1]

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Received for publication June 25, 1936

Although a tremendous amount of work has been done on the subject of bacterial variation, the major part is concerned with the description of certain variants, interesting chiefly for their morphological, colonial, biochemical, or immunological peculiarities. There have been but few attempts to study the fundamental nature of bacterial variation and such hypotheses as have been advanced differ widely from each other with the result that practically every type of variation observed in higher forms of life has been suspected of occurring in bacteria.

Neisser (1906) and Massini (1907) working with *Escherichia coli-mutabile*, stated that lactose-fermenting variants arose from the parent culture by a process resembling mutation. This is in agreement with the recent work of Lindegreen (1935 a and b; 1936). Penfold (1912) working with Twort's lactose-fermenting bacillus believed that it might become stabilized by constant selection. Stewart (1926) constructed a very interesting hypothesis of asexual character segregation, although he admitted that conjugation might occur in the colonial papillae. A similar hypothesis was presented by Reed (1933). Mellon (1925 a and b; 1927), in a large series of papers on this subject offered evidence that conjugation (chiefly autogamous) may occur. Other sexual phase and life cycle theories have been presented by Fuhrman (cited by Hadley, 1927), Hort (1917), Löhnis (1921), Leiske

[1] **Submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.**

(cited by Hadley, 1927), Eisenberg (1918), Enderlein (1925), and Almquist (1911; 1922).

In dealing with bacterial variation, we are confronted with a permanent change in the heritable characters of protoplasm. Such a problem is essentially a genetic one and its solution would seem to be best approached through statistical methods. However, because of the scarcity and relative unpredictability of dissociants as they ordinarily occur, and because of the complexity of the conditions under which they are encountered, statistical studies are difficult to undertake. Yet it is possible that such studies may yield information concerning the mechanics involved in the origin of a dissociant cell. We may discover that variation is wholly an accidental and unpredictable phenomenon; or perhaps that it is governed entirely by environmental conditions. We may, on the other hand, find that such phenomena follow well established laws which operate independently of external stimuli.

Salmonella aertrycke was selected as a suitable organism for the study of variation since it is a common and well-studied pathogen possessing simple morphological characteristics.

In the course of a study of the above-mentioned organism, a number of variants were encountered which behaved differently from the ordinary bacterial variants. The chief difference was that these variants, upon continued cultivation, always gave rise to two types of colonies in easily-countable proportions. Such variants promised to be useful instruments for the investigation of bacterial variation for they lent themselves admirably to statistical studies. These variants are referred to as unstable variants.

EXPERIMENTAL

A simple method of obtaining a large variety of dissociants is by aging a broth culture of bacteria. Thus, when a broth culture of *S. aertrycke* which was allowed to age at 37°C. for several weeks was streaked on an agar plate, it was found to yield an abundance of colonial variants. Most of these colonial variants when substreaked on agar yielded, as would be expected, colonies, all of

which were identical with the parent colony in every observable respect. Occasionally, however, as in the case of the variant colony labelled R3, an entirely different condition was obtained. This variant was characterized by a rough colony which when substreaked gave rise to both rough and smooth colonies on a 24-hour agar plate. The smooth offspring when substreaked yielded all smooth colonies. The rough offspring, however, again gave rise to both rough and smooth colonies on subculture. An attempt was made to obtain the rough form in pure culture by constant selection and subculture of the rough colony. Although this procedure was continued almost daily for about three years, the rough colony always gave rise to both smooth and rough colonies. It was clear that this rough variant was unstable, i.e., on subculture always gave rise to a second variant type as well as to the original colony type.

Evidence was obtained by microscopic study that the instability of this rough variant was due to the presence in the rough colony of two types of cells, a long filamentous form and a few short bacilli. When an individual short rod was fished from the rough colony by means of a micro-pipette and planted on agar, it gave rise to a smooth colony containing only short rods. When an individual long rod was subcultured by micro-isolation, it yielded a rough colony which again showed some short forms among a predominance of long forms.

Thus, the long rods must have produced a certain number of short rods in the course of development of the unstable colony. It seemed therefore that the long rod is an unstable cell which may develop into an unstable rough colony, and that these unstable cells constantly give rise to the short rods which are stable cells and may develop into the stable smooth colony.

In order to determine whether any decreased tendency to give rise to the stable smooth colony resulted from constant selection of the unstable rough colonies, the following quantitative method was employed. A rough, unstable colony was streaked out on agar to give a large number of well-isolated colonies. After incubation at 37°C. for about 24 hours, such a plate exhibited the two types of colony, R and S. The R and S colonies on part of

the plate were counted separately and the abundance of R was expressed either as a ratio of R:S or better, as the percentage of R colonies in the total of all the colonies (R + S) counted. The percentage so obtained was 81 per cent R and 19 per cent S. Careful estimation of the percentage on plates streaked with R colonies during successive daily subculture revealed no tendency towards either an increase or a decrease in the percentage of R colonies. The percentage then, was constant from day to day, and predictable within certain limits. Since the ratio of R:S colonies may be taken to indicate the ratio of R:S cells in the parent unstable colony, it may be inferred that this latter ratio was constant in any 24-hour rough colony. Thus, the production of R and S cells by the R cell which gave rise to the rough colony was apparently based upon a definite genetic mechanism. Moreover, the phenomenon of unstable variation resembled ordinary bacterial dissociation except that it occurred more rapidly. It was believed that the rapidity and regularity with which unstable colonies produced variants might make them a desirable instrument for the study of the mechanism underlying dissociation in general.

OTHER UNSTABLE VARIANTS[2]

In addition to the unstable variants described above, several others have been encountered.[3] Most of the unstable variants were isolated from old broth cultures. Such variants are not rare. Fifteen out of a total of 45 distinct colonial variants of *S. aertrycke* proved to be of the unstable type. Nor are they limited to any particular colonial type, for they included smooth, rough, mucoid, flat and intermediate forms. A study of these unstable variants showed that each produced a characteristic percentage of unstable and stable colonies.

In order to conserve space, only a few of the unstable variants of *S. aertrycke* will be discussed. (In the labels of the different

[2] These variants were studied almost exclusively with respect to colony form since for the purposes of this experiment this provided a simple criterion for determining character change.

[3] Although most of this work was done with unstable variants of *Salmonella aertrycke*, some unstable variants were obtained from other species such as *Salmonella enteritidis* and the Friedlander bacillus.

variants, the letters denote colonial properties, as R for rough, F for flat, M for mucoid, O for opaque. Combinations of letters imply that the colony exhibits both characteristics.) R3 is characterized by a rough, unstable colony[1] which gives rise to a certain percentage of small, smooth, stable colonies (fig. 1); R4 is a small, rough, unstable colony which yields a large, rough

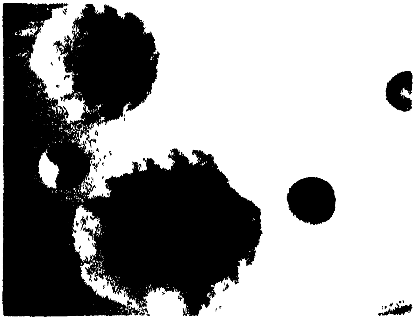

Fig. 1. R3, the large colony is the unstable

Fig. 2. R4, the small colony is the unstable

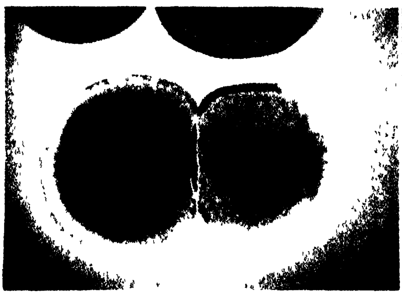

Fig. 3. OF, the dark colony is the unstable

Fig. 4. RF, the convoluted colony is the unstable

FIGS. 1 TO 4. PHOTOGRAPHS OF TYPICAL UNSTABLE COLONIES TOGETHER WITH THEIR STABLE OFFSPRING

stable colony (fig. 2); OF is an opaque, unstable colony which yields a stable flat form (fig. 3); RM is a mucoid colony which yields a rough stable form. RF is a rough unstable colony which yields a stable flat colony (fig. 4). All the variants of *S. aertrycke*

[1] "Unstable colony" is a convenient term for what is probably a mixed colony arising from an unstable cell

described in this report had the same biochemical, staining and flagellar agglutination properties as the original smooth strain of *S. aertrycke*.

FIXITY OF RATIOS OF THE TWO COLONY TYPES OBTAINED FROM UNSTABLE VARIANTS

These unstable organisms were carried for various periods of time up to about two years. During this time colonies of each unstable strain were substreaked on agar plates from day to day

TABLE 1

Percentage of unstable colonies obtained on substreaking an unstable colony

INTERVAL DATES	UNSTABLE VARIANT				
	R4	R1	OF	R3	RF
January, 1934 March, 1934		80 ± 5		81 + 2	
May 16, 1935 May 21, 1935	99 +	82 ± 3	77 + 1	80 ± 2	87 ± 1
May 31, 1935 June 15, 1935	99 +	78 ± 1	75 ± 7	79 ± 8	86 ± 8
June 15, 1935 June 25, 1935	98	79 ± 6	76 ± 6	79 ± 1	81 ± 6
December 6, 1935 December 30, 1935	97		77 ± 6	83 ± 6	81 ± 7
Total mean	98	79 ± 5	76 ± 5	81 ± 5	83 ± 5

and the ratios of the two types of colonies obtained from each estimated. This was done to determine whether the proportions of the two types of colonies were constant and predictable from day to day or whether there was any observable shift in these proportions in the course of time.

A complete list of every percentage determination for each of the organisms would require an excessive amount of space. Instead, the average ratio of counts taken during certain intervals are shown in table 1. The average deviation from the mean is given for each set of counts. The deviations from the mean give some information concerning the daily variations of each culture.

Inspection of table 1 will show that in most instances there was little tendency for the percentage figures either to increase or to decrease. The most significant variations were the daily deviations from the mean average.

It was important to determine whether these deviations were the result of technical errors or whether they were actually true variations in the proportions of the two types of organism present in an unstable colony.

This question can be answered by determining the ratios of the two colony types on a large number of plates streaked from a single colony. Theoretically all such plates should show the same percentage figures. Any deviation from the average percentage is due to technical error. If deviation values on plates streaked from several individual colonies are much greater than those on plates streaked from one single colony, then part of that deviation must be due to inherent differences in the proportions of the two types of cells in the several unstable colonies.

TABLE 2

Comparison of the average deviation from the mean when several substreaks are made from the same colony or from several colonies

VARIANT	MEAN	A.D.M. FOR SINGLE COLONIES	A.D.M. FOR SEVERAL COLONIES
R3	82	4	4
RF	76	7	6
OF	74	3	6

In this experiment a single unstable colony was triturated thoroughly with a loop. Twelve plates were streaked with this material. The plates were then incubated and inspected in the fashion described and the results listed in table 2.

The column labeled "Mean" gives the average of the percentage of unstable colonies on 12 plates streaked from a single unstable colony; the next column represents the average deviation from the mean of each of the 12 counts on plates streaked from one colony; the last column represents the average deviation from the mean of each count made on plates streaked from separate colonies (during serial daily subcultures).

The error involved in determining the percentage figure for certain of these variants is much greater than for others. In general the deviation in estimating several plates each streaked from a separate colony approximates that encountered in estimating several plates which have been streaked from a single colony. Hence, it seems possible to conclude that the variations of the percentage of unstable colonies derived from separate unstable colonies falls within the experimental error.

It appeared from the preceding experiments that variations of the percentage figures even over long periods are imperceptible, and that the composition of the colonies is constant within the experimental error. Having established these facts, it was possible to proceed with other experiments and recognize significant deviations from normal behavior.

In the following experiments an attempt was made to study the influence of various environmental conditions upon the percentage of stable and unstable colonies arising from an unstable colony.

THE EFFECT OF VARIOUS ENVIRONMENTAL CONDITIONS ON THE ABILITY OF UNSTABLE COLONIES TO YIELD STABLE AND UNSTABLE COLONIES

1. The effect of aging. A typical unstable colony was selected from each of the variants and inoculated on the surface of agar plates. These plates were incubated at 37°C. in a moist chamber. At various intervals parts of the colonies which resulted were substreaked on agar plates to determine the percentage of stable and unstable colonies which they yielded. Table 3 illustrates the results obtained.

Most of the colonies showed an ultimate disappearance of the ability to yield unstable colonies on subculture. If we are to assume that, in the unstable colony, unstable cells are constantly giving rise to stable cells, then ultimately the stable cells would far out-number the unstable cells.

Throughout this experiment no permanent transmissible change was observed, for whenever an unstable colony was taken from an aged culture and streaked on fresh agar, the 24-hour unstable

colonies yielded the original percentage of stable and unstable colonies.

2. The effect of incubation at lowered temperature. Typical unstable colonies were streaked on ordinary nutrient agar plates and left at room temperature (about 23°C.) for 72 hours, at which time the colonies attained about the same size as those incubated

TABLE 3

The percentage of unstable colonies arising from agar cultures of unstable variants aged for varying intervals

DAYS	UNSTABLE VARIANT			
	R4	RF	R3	OF
1	88	74	89	67
2	97	93	93	
4	96	94	1	27
5	95	85	0	22
17	0	0	0	19

TABLE 4

Percentage of unstable colonies arising from unstable colonies grown in various environments

ENVIRONMENT	UNSTABLE VARIANT			
	R3	OF	RF	R3
72 hours, 23°C	98	61	70	46
Phenol agar	99	82	95	90
Lithium chloride	99	84	84	82
4 per cent peptone	99	79	67	69
Blood	93	79	92	73
E.M.B	99	57	76	85
Endo	99	76	93	11
Plain nutrient agar	97	77	81	83
Daily mean	98	76	83	81

at 37°C. for 24 hours. These colonies were then streaked on agar plates and the percentage of unstable colonies arising from them determined.

The only consistent effect produced by growth of unstable colonies at lowered temperature was a reduction of the percentage of unstable colonies arising from the R3 colony (see table 4).

3. The effect of various chemicals. Phenol: Unstable colonies were streaked on agar containing various quantities of phenol. Only those concentrations (0.1 to 0.2 per cent) permitting exceedingly poor growth were considered, in order to make certain that the phenol had exerted a maximal effect.

As may be seen from table 4, strong concentrations of phenol, surprisingly enough, had little effect upon any of the unstable colonies except for an elevation of the percentage of unstable colonies obtainable from the RF colonies.

Lithium chloride: The same procedure was followed with lithium chloride as with phenol, and this salt even in concentrations resulting in marked inhibition of growth produced no significant effect (see table 4).

In addition to the above media unstable organisms were grown on the following media: Agar containing 4 per cent peptone, or twice the usual concentration of peptone, blood agar (these two providing an enriched medium), and finally Endo and eosin methylene-blue plates, in order to determine the effect of sublethal quantities of common dyes. Of the last four media mentioned the only one that exerted any apparent effect was the Endo medium.

4. The effect of pH. Unstable variants were streaked on agar of pH ranging from 5.6 to 9.6. The plates were then incubated for 24 hours at 37°C. after which time typical unstable colonies were fished from them and streaked on fresh agar of the usual pH of 7.6 to determine the percentage of unstable colonies they would yield.

As seen in table 5, unstable colonies grown on agar of varying pH did not differ very strikingly from colonies grown on agar of the usual reaction of pH 7.6 in their ability to yield unstable colonies despite the fact that marked inhibition of growth occurred when the reaction was either 5.6 or 9.6.

In all the experiments with changed environment, whenever a changed ratio appeared, the colonies concerned were subcultured several times to determine whether that change was permanent. In no case did a permanent change result, for on subculture in normal environment the usual ratio prevailed. Apparently the

environmental influence did not produce any permanent change in the tendency of unstable variants to produce stable colonies.

5. Behavior of unstable variants in broth. Although the chemical and physical changes of the environment as outlined above did not produce marked changes in the percentage of stable and unstable colonies obtainable from unstable colonies, it was found that the simple expedient of growing these variants in broth almost invariably produced a very striking reduction of the percentage of unstable colonies.

Let us consider a specific example. It has been previously stated that an agar growth of the strain R3 yielded 81 per cent unstable colonies when substreaked on agar. A broth culture, on the other hand, gave only about 25 per cent unstable colonies

TABLE 5

The percentage of unstable colonies arising from unstable colonies grown on agar of different pH

pH	UNSTABLE VARIANT			
	RF	FM	R3	R4
5 6	89	84	85	99+
6 0	96	70	82	99+
7 6	81	77	83	97
9 0	89	75	92	99+
9 6	81	70	85	99+

when streaked on agar. However, when an unstable colony derived from a broth culture was substreaked on another agar plate the normal percentage, or about 81 per cent, of unstable colonies was obtained. The phenomenon of the reduction of the percentage of unstable colonies obtainable from broth was repeatedly obtained, but each time, substreaking an unstable colony on a second agar plate always gave the standard ratio of unstable colonies.

The results for R3 and other unstable variants are summarized in table 6. In each case the unstable variant was cultured alternately on broth and agar for about twelve generations. The column "1st agar subculture" gives the average percentage of unstable colonies obtained from 24-hour broth culture; the

column "2nd agar subculture" gives the average percentage of unstable colonies found by streaking the agar colonies obtained by subculturing the broth.

The production of unstable colonies was markedly reduced in broth. Some variants, as R3 and RF, were affected much more than others.

By streaking a broth culture at regular intervals after inoculation with an unstable colony it was found that the most rapid lowering of the percentage occurred during the early part of the growth phase, before the broth had become turbid.

Since the percentage of unstable cells in a colony arising from an unstable cell was a constant transmissible character it was employed in these experiments as a quantitative indicator of genetic change. In no case did a percentage change due to altered environment effect a detectable genetic change.

TABLE 6

The percentage of unstable colonies obtained from 24-hour broth cultures of unstable variants on first and second agar subcultures

UNSTABLE VARIANT	1ST AGAR SUBCULTURE	2ND AGAR SUBCULTURE	STANDARD PERCENTAGE OF UNSTABLE COLONIES PREVIOUSLY OBTAINED ON AGAR
	per cent	*per cent*	*per cent*
OF	69	77	77
RF	6	65	58
R4	46	97	98
R3	25	85	80

Hence, in all probability even the marked percentage shift encountered on growing these variants in broth was nothing more than a temporary phenomenon, due perhaps to a more rapid growth rate of the stable cell in broth and not to any modification of the genetic mechanism of the unstable cell.

DISCUSSION

Multiplication of bacteria is concerned with the transmission of various characteristics from cell to cell. A single smooth cell for instance, on repeated division gives rise to billions of smooth cells, indistinguishable from the parent cell. This is manifestly a

genetic or hereditary phenomenon. At times some of the cells in a bacterial culture exhibit new and transmissible features. Such modifications are known as variations. Changes of this type involve a change of genetic material in a fashion which at the present time is not understood. Unfortunately, bacterial variation usually occurs with such irregularity and infrequency as to preclude any possibility of a reliable statistical study which is the key to most genetic problems.

The so-called unstable variants described in this paper offer advantages unattainable with ordinary variants in attempting a statistical approach to the phenomenon of bacterial variation. In the first place, the number of variant offspring is considerable and may easily be counted. Secondly, the offspring are abundantly produced on simple media, thus obviating the necessity of modifying the environment, as is common in most studies of variation. These advantages made it desirable to conduct a detailed investigation of the behavior of unstable strains.

Instability characterized a high proportion of the variants encountered in the course of this research—about 14 out of 40 in *S. aertrycke* alone. This fact indicates that cultural instability must be a common phenomenon. Possibly such variants would be detected much more frequently were it not for the fact that they tend to be replaced by the stable form when not carefully selected. Instability is not limited to any particular type of variant, but may occur in different colonial forms such as mucoid, rough, and flat. Moreover, cultural instability is not limited to the species with which most of this work has been done. Several unstable strains of *S. enteritidis* and of the Friedlander bacillus have been isolated in connection with this study. Twort's lactose-fermenting variant of *Eberthella typhi* is of a similar nature. Recently, several unstable cultures of *Staphylococcus aureus* have been isolated by R. Thompson (personal communication).

There is evidence that each unstable culture is a mixture of two types of bacterial cells, those breeding true, i.e., yielding a pure, stable culture,[5] and those constantly giving rise to a mixed culture.

[5] The term "pure" or "stable" culture is employed in a relative rather than an absolute sense. It implies that such cultures rarely exhibit variation during daily subculture on agar plates.

It has been impossible to obtain a pure culture of unstable cells, i.e., a culture of unstable cells free from stable cells either by plating or by single cell isolation. In most cases, the stable variant differs only slightly from the unstable variant, i.e., an unstable mucoid yields a stable mucoid; an unstable rough yields a stable rough. Occasionally, however, the unstable variant has properties which differ markedly from those of its stable offspring. In R3, for example, a very rough, large unstable colony gives rise to a very smooth, small, stable colony. This is an abrupt "saltation" from one colony type to another without the occurrence of intermediates.

In most instances these variants were carried on agar by subculturing unstable colonies. It is likely that such a colony is a mixture of stable and unstable cells, so that on streaking a colony of this sort, one again gets two types of colonies. The observation that the ratio of one type of colony (unstable) to the other type (stable), is constant is especially significant. It implies that the ratio of the different types of cells arising from an unstable cell is constant. Here we have a cell which as it multiplies constantly splits off cells of another type.

These variants were subcultured on agar in the manner stated for various periods of time, during which the percentage ratios were determined on each subculture. There was no detectable tendency to an increase or decrease in the ratio even after two or three years, despite constant selection of the unstable type.

Unstable variation therefore appears to be controlled by a definitely fixed, inherent mechanism.

Effect of changed environment

These variants were exposed to various environmental conditions, such as changed pH, inhibiting doses of chemicals, dyes, enriched media, and different temperatures. In most cases, even when the changed environment had a marked effect upon the size and appearance of the growth, it was found that the percentages of stable and unstable cells were only slightly changed. Whenever such a change did occur it was only temporary, for the old, established ratio was immediately regained on subculture to normal environment.

Only in broth did a consistent change in percentages of unstable colonies occur. The altered percentage of unstable colonies obtained from broth cultures was not associated with a permanent change in the ability of the strain to yield unstable colonies, for a broth culture plated on agar gave colonies which on subculturing were quantitatively indistinguishable from the usual agar colonies of that variant. In other words, broth did not effect a permanent change in the genetic properties of the unstable variants, and there is evidence (Deskowitz and Shapiro, 1935) that the observed change was due to a more rapid growth of one of the two variants (usually the stable) in broth. This would result in a higher final percentage of the type having the more rapid growth rate.

The final percentage of the two types in any medium is probably a resultant of two factors: first, the actual rate with which stable cells split off from unstable cells—this might well be denoted the genetic ratio; and second, the relative growth rates of the two cell types in any given medium.

The mechanism of unstable variation

Studies of genetics have shown that heritable variations may result from sexual recombination, chromosome changes, gene mutations, and life cycle changes. These different possibilities will be briefly considered as they may apply to the phenomenon of bacterial variation.

Sexual phase. If ever there were a possibility of establishing the existence of a sexual phase in bacterial variation, unstable variants should offer it. It could not be argued here that only a few cells dissociate and therefore it is very difficult to encounter sexual forms. In an unstable culture variation is constantly and abundantly occurring. Hence, if a sexual phase preceded the formation of a new cell type, it should be found much more easily than in common dissociation.

However, no satisfactory evidence for the occurrence of a sexual phase was encountered in the strains described. The occurrence of variation does not necessarily imply a sexual phase. Punkari and Henrici (1933) have shown, in the case of yeasts, that ordinary dissociation may occur in the complete absence of the sexual phase.

Gene mutations. Only a few years ago the phenomenon of unstable variation, because of its rapidity, predictability, and reversibility would have been excluded from the realm of true gene mutations. Recently, however, as a result of studies on the effects of radiation, gene mutations were discovered which parallel every feature of unstable variation. Demerec (1932; 1934), studying the effect of temperature changes or exposure to x-rays discovered certain strains of *Drosophila* which mutated repeatedly, yielding up to 50 per cent mutations with respect to one particular character. The occurrence of these mutations was statistically predictable and in many cases reversible. This phenomenon, sometimes known as recurrent or unstable mutation, seems to be an exact counterpart of unstable bacterial variation. The discovery of unstable genes has enlarged our concept of mutations considerably so that the unstable phenomenon can be easily included in this class.

The possibility of chromosome changes is considered in detail by Lindegren (1935 a and b; 1936) who offers evidence for the existence of chromosome-like bodies in bacteria.

While it is yet impossible to tell which type of variation in higher forms corresponds to unstable variation among bacteria, it seems that this phenomenon most closely parallels gene mutation as we now know it.

The relationship between unstable variation and ordinary dissociation

The only important difference noted between the two would seem to be the rate at which variants are given off.

It is known that some bacteria have a greater tendency to vary than others. The smooth form of most species varies more readily than does the rough. Even among the unstable organisms the tendency to vary differs greatly. With a variant M (not hitherto discussed), it is 41 per cent, with R3, it is 19 per cent, and with R4, it is less than 2 per cent. Hence, M is more unstable than R3, and R3 is more unstable than R4. The variant R4 with its ratio of 98.2 approaches the limit at which one may readily recognize an unstable organism. It may be that the so-called pure or stable organisms are in reality unstable,

but the percentage of variants given off are so few that they escape detection during ordinary cultivation. It seems probable that common bacterial dissociation and unstable variation are expressions of the same phenomenon, but differ only quantitatively. A parallelism between bacterial dissociation and unstable variation exists in the observation that in both cases the variant form appears more abundantly in broth than on agar, and furthermore, appears more rapidly in large volumes of broth than in small volumes.

The experimental work included in this report suggests a tentative hypothesis which may be stated as follows:

Bacterial variation is based upon a fixed genetic mechanism which appears to operate independently of external environment. Each organism has an inherent potentiality for producing a variant form once in a given number of divisions. This may be referred to as the primary or genetic ratio. However, since the final ratio of the variant and the parent type in the culture will also be determined by the relative growth rates of the two types of organisms, it may be readily seen that any environmental change which alters the relative growth rates will affect the ease of detection of the variant form.

Such a concept does not preclude the possibility of occasional mutations resulting from environmental change. Mutations have been produced by x-rays and other environmental stimuli in the case of higher forms. However, in the course of our study of unstable bacterial variation, we have never detected a heritable change as a result of environmental influence.

SUMMARY

Several unstable variants have been isolated from *Salmonella aertrycke* and other bacterial species. An unstable variant is characterized by the property of constantly giving rise not only to the parent colonial form but also to a second colonial form. Under fixed environmental conditions, the ratio of the new variant to the parent type always remained constant despite repeated selection of the parent type. Altered environment, in certain instances, produced a temporary change in this ratio, but on

reculturing in the original environment the ratio always reverted to its original value. It appeared, therefore, that the potentiality to produce variants is an inherent property of the bacterial protoplasm independent of environment. The possibility that ordinary dissociation and unstable variation differ only quantitatively, is discussed.

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A CONTAGIOUS TUMOR-LIKE CONDITION IN THE LIZARD (ANOLIS EQUESTRIS) AS INDUCED BY A NEW BACTERIAL SPECIES, SERRATIA ANOLIUM (SP. N.)

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Received for publication October 16, 1936

In the spring of 1935 our attention was attracted by a tumor-like condition observed in a species of Cuban lizard, *Anolis equestris*. These lizards had been sent to the Museum of Natural History and kept in the laboratories for some time. In the course of several months the disease had successively appeared in several individuals living in the same cage. New cases have appeared consistently up to the present time.

The condition was characterized by ovoid nodules of a size varying from 2 x 2 mm. to 10 x 25 mm. They were located in various regions of the body, such as toes, vocal pouch, forelimb, elbow, etc. They were felt under the skin and were loosely attached to the surrounding tissues. It was not a rare occurrence to find several nodules developing simultaneously or successively in the same individual. The process of growth of the nodules was rather slow. However, in one case a tumor-like mass arose in the lower jaw and attained a size of 18 x 3 mm. in 10 days. No clear evidence has been obtained to date that any of the lizards died as a direct result of the natural disease.

A number of the nodules were removed for microscopic study. The nodules were usually rather hard and upon sectioning, all of them consisted of a whitish, soft and viscid tissue. The central part was yellowish and necrotic. This tissue was surrounded by a rather strong fibrous capsule. Tissues around the mass were found to be somewhat hemorrhagic.

Microscopic examination in fixed and stained sections and

fixed or supravitally stained smears showed the healthy viscid tissue to be formed by the following cells: non-phagocytic lymphocyte-like cells, larger phagocytic cells of the monocyte and clasmatocyte type, fibroblasts and a few giant cells. Mononuclear eosinophilic cells were also seen. The fibrous capsule was heavily infiltrated by mononuclear cells. These were especially conspicuous around the vessels. A pleomorphic, gram-negative bacterium was abundantly present in every case. Evidently we were dealing with a chronic infection of bacterial origin. In fact cultures of the nodular material on agar immediately yielded abundant pure cultures of an organism. The cultural and morphological characters of this organism are expressed as follows.

MORPHOLOGY

Pleomorphic organism. The most common form in broth (figs. 1 and 2) is a slender rod 1 to 2 micra in length and 0.2 to 0.4 in breadth; sides parallel, axis straight; arranged either in clusters and palisades or singly and also in pairs. Other individuals are 4 to 5 micra in length, curved, of uneven diameter and even club-like. Long forms, 10 to 15 micra or more, are also seen and these seem to be surrounded by a capsular material. On the other hand, small coccus-like elements are not infrequent.

On agar the morphology is different in smooth as compared to rough colonies. In the smooth colony (fig. 3) there is a predominance of long elements detached from each other forming only small clusters; filamentous forms are common. Rough colonies (fig. 4) are mostly made up of the more uniform small types which gather in large and dense clusters. Variation in the depth of staining of different individuals with methylene blue occurs in both forms. However, this phenomenon is more marked in the short bacteria of the rough colonies. Bacteria from smooth colonies often show unstained areas in their bodies. Bipolar staining is not infrequent. In young spreading colonies on agar plates, bacteria from the peripheral parts show a morphology similar to that of bacteria from smooth colonies, whereas bacteria from the central parts resemble those from rough colonies.

The organism is motile, gram-negative and non-acid-fast.

AGAR PLATE

If the medium is seeded by a stroke, a marked spreading of growth results during the first few hours (fig. 5). However, the medium is not completely invaded and the phenomenon is not like the "swarming" of *Proteus*. After 24 hours the growth becomes thicker with a crenated or fimbriated contour.

If the medium is uniformly seeded (fig. 6), a growth with a marked confluent tendency results. After 24 hours at 37°C. isolated colonies are low, convex, entire or slightly undulate, translucent, butyrous, glistening, and smooth, with a tendency toward radial structure (fig. 7). Their size is from 1 to 2.5 mm. in diameter. Later the colonies become larger, frankly striated and wrinkled, showing opaque, radiating folds with irregularly crenated edges, and a rougher texture. However, some colonies retain their smooth character and intermediate forms are also noted. Cultures produce a penetrating acid smell.

AGAR SLOPE

24 hours at 37°C. Abundant, confluent, raised, whitish, butyrous, glistening, with entire or undulate edges (fig. 8). Later changes are similar to those occurring in agar plates. A slight pink coloration may develop at the lower and thicker part of the growth. If the organism is seeded in the water of condensation a rapid growth occurs, ascending 3 or 4 cm. during the first hour but no real "swarming" or complete invasion of the medium is observed.

Gelatin stab; 24 hours at 20°C. Rapid growth. Infundibuliform liquefaction from the first hour. Thick soft pellicle formed, *6 to 10 days*, total liquefaction, blackish sediment formed.

Broth; 24 hours at 37°C. Moderate growth with uniform turbidity. More abundant growth in the upper layers. *Three to 6 days*, marked increase of the growth and formation of a pellicle which disintegrates and adheres to the tube wall, forming a conspicuous ring. A sediment is formed; a faint fluorescent yellowish coloration develops.

Glycerol broth. Same as in normal broth.

Glucose broth, 2 per cent; 24 hours at 37°C. Same as normal

broth. *Six to 10 days at room temperature;* marked turbidity, clear yellowish coloration.

Peptone water; 24 hours at 37°C. Same as normal broth. *Six to 10 days at room temperature;* marked turbidity, medium darkened, blackish sediment formed.

Peptone water with 2 per cent glucose; 24 hours at 37°C. Same as normal broth. *Six to 10 days at room temperature;* marked reddish pigmentation of the upper layers first, and later invading the lower depths of the medium.

Rabbit-blood agar plates; 24 hours at 37°C. Abundant growth. Broad zone of hemolysis around each colony. *Forty-eight hours;* total hemolysis.

Loeffler serum; 24 hours. Abundant glistening growth. Beginning of liquefaction. *Three days;* advanced liquefaction.

Potato; 24 hours at 37°C. Abundant, butyrous, glistening, raised growth. A pink coloration may develop. *Four to 10 days at room temperature;* increase of density in growth and pigmentation. Pigment diffuses through the medium.

Glycerol potato; 24 hours at 37°C. Same as in plain potato. Pigment production more marked, especially if the medium is seeded from another pigmented growth on glycerol potato.

Resistance. Cultures are still viable at least after two months at 5 to 10°C. Heating at 60°C. for 20 minutes kills the microorganism.

Metabolism. Aerobe: no visible growth in boiled broth with or without glucose under vaseline layer, after three weeks. No growth in broth or agar in a vacuum.

Biochemical. Glucose, levulose, sucrose, mannitol, maltose, galactose, and salicin are fermented with production of acid. Dextrin, lactose, inulin, dulcitol, xylose, and arabinose are slightly attacked or not at all. Gas can be produced from certain carbohydrates such as glucose, levulose, sucrose and mannitol, but in small amounts. Traces of it can be detected in other carbohydrate-containing media. Gas production does not seem to be a regular phenomenon.

Plain milk, 24 hours. Clotting. Four to 10 days. Digestion of coagulum. No pigment formation.

Litmus milk, 24 hours. Clot; partial discoloration of litmus. Ten to 15 days. Complete digestion of the coagulum.

Kahn's medium. No growth.

Indol −.

SH_2 −.

Nitrates not reduced to nitrites.

Ammonia +.

Grows well at 20°C. Growth more abundant at 37.5°C. Practically no growth at 10°C.

Pigment production. As already indicated, the bacterium is capable of developing a pigment which gives diffuse coloration to the medium. The pink coloration is best shown on glycerol potato. The reddish coloration is best shown in peptone water with 2 per cent glucose, the yellow one in glucose broth, and the black one in the sediment of liquefied gelatin and peptone water. As far as our work goes, the pigment does not seem soluble in alcohol, chloroform, ether and acetone, but is soluble in water. Irregularities in pigment production have been observed. For reasons still unknown some cultures fail to develop pigmentation.

EXPERIMENTAL DISEASE

Injection of the bacterium from isolated bacterial cultures in broth or agar were made into healthy individuals of the same species of lizards from which the bacterium was isolated. This produced a disease which in many ways was comparable with the natural disease. Details of these experiments will be given in a subsequent paper.

COMMENT

From the above data there is no doubt that we are dealing with a contagious disease of a rather chronic type in *Anolis equestris*. The disease is produced by a bacterium. This is shown by the consistency with which this bacterium was isolated from lesions of every animal studied during several consecutive months, and also by the reproduction of the experimental disease in the animal by inoculation with the same bacterium. Typical lesions were produced from which the causative bacteria were again isolated.

As far as the final classification of the organism is concerned, no final conclusions have yet been reached, since the organism has not been thoroughly studied, especially from the serological standpoint. However, the strong pink pigmentation which the organism produces when grown on plain and glycerol potato, and the other fainter colorations which develop in the media, would allow one to make a temporary classification including the microorganism in the family *Bacteriaceae*, tribe of *Chromobacterieae*, genus *Serratia*. This genus has been created to comprise those bacteria producing a red or pink pigment. Moreover, the other characters of cultures of the organism seem to fit well with those of this group. Hence, this organism is described as a new species, *Serratia anolium*. Nevertheless, more work is needed to establish the absence or presence of characters which might tend to place the bacterium near the *Pseudomonas* or still other genera. On the other hand, there is considerable confusion regarding bacteria belonging to the tribe *Chromobacterieae*, and the fact that "*Serratia anolium*" would be one of the few if not the only described species of this genus possessing pathogenic properties, at least for cold-blooded animals, would warrant a more detailed study of the organism. The bacillus seems to dissociate easily and a study of its variations in virulence, pigment production, and other characters is now under way.

SUMMARY

A new bacterium has been studied which is the causative agent of a contagious disease in *Anolis equestris*, characterized by tumor-like lesions of a rather slow evolution and seldom directly fatal to the animal. The organism is a chromogenic, gram-negative, non-spore-forming, and motile rod, which has been classified temporarily as belonging to the group of *Chromobacterium*, genus *Serratia*. The denomination of the new organism would be *Serratia anolium*. Injection of this organism into healthy *Anolis* lizards results in a condition similar to the natural disease.

PLATES

PLATE 1

Explanation of Figures

Fig. 1. Smear from broth culture 24 hours old. × 530.
Fig. 2. Smear from broth culture 24 hours old. × 1000.
Fig. 3. Smear from a 24 hour sub-culture in agar of a smooth colony. × 530.
Fig. 4. Smear from a 24 hour sub-culture in agar of a rough colony. × 530.

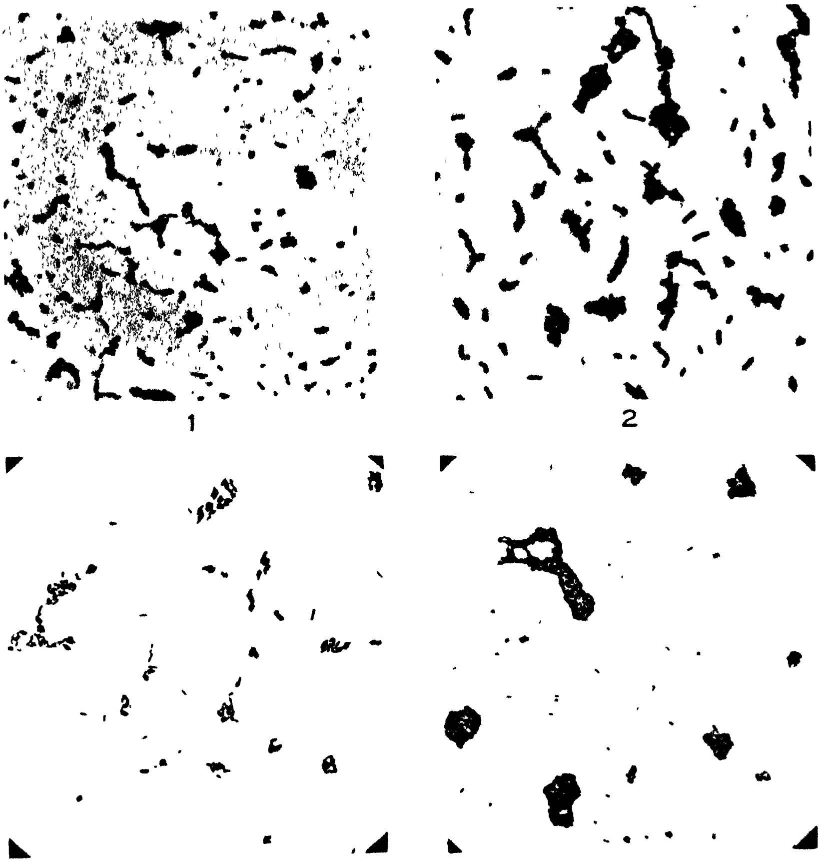

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PLATE 2

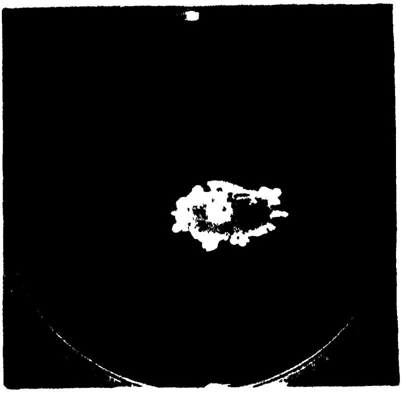
Explanation of Figures

Fig. 5. Spreading growth obtained after 24 hours of seeding the agar plate on a single point of its surface. The same area of growth, except not as thick a growth, was obtained 5 hours after seeding. × 0.5.

Fig. 6. Agar plate 48 hours old, uniformly seeded. × 0.5.

Fig. 7. Colonies 5 days old on plain agar. × 5.

Fig. 8. Twenty-four hour agar slope. × 0.5.



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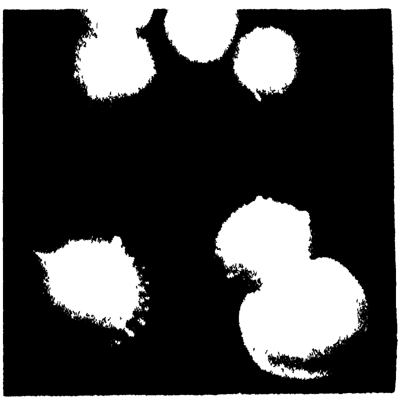

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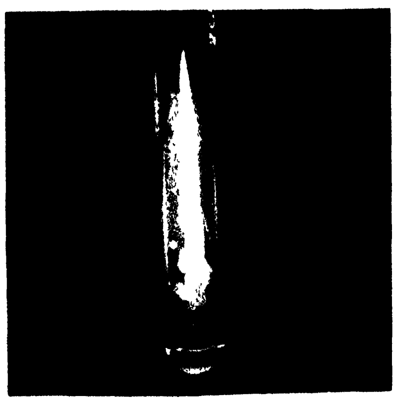

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THE LIPOLYTIC ACTION OF STAPHYLOCOCCI ON SOME PURE TRIGLYCERIDES

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Received for publication October 19, 1936

In 1901 Eijkman devised a plate method to test for the presence of bacterial lipase using as a substrate beef tallow over which inoculated agar was poured. A positive test was indicated by the appearance of opaque spots in the tallow due to the formation of fatty acids. Staphylococci were included among the organisms designated as forming a lipase.

Wells and Corper (1912), using toluene water emulsions of large amounts of bacterial growth, tested five different organisms on ethyl butyrate, triacetin and olive oil. Staphylococci were the most actively lipolytic followed in order by *Pseudomonas aeruginosa*, *Escherichia coli*, *Shigella dysenteriae* and *Mycobacterium tuberculosis*. They state that "presumably the same enzyme attacks all three esters." They also concluded that Eijkman's plate method shows only the most active degrees of lipolysis and is solely a relative test for lipase. They were able to demonstrate lipolytic activity in their experiments on the part of organisms which gave negative tests by the plate method. Their method of estimating the amount of fat hydrolysis consisted in titrating the mixtures of fats and emulsions of organisms at intervals using N/10 NaOH and phenolphthalein.

Orcutt and Howe (1922) reported an investigation of certain strains of staphylococci which were hemolytic only in the presence of fat. This was determined to be due to the production of fatty acids through action of the bacterial lipase.

The experiments herein reported were undertaken to determine if there was any relationship between the lipolytic activities

of staphylococci and their virulence, the type of lesion from which the pathogenic strains were isolated, or the source of the non-pathogenic strains. Further, we desired to know if lipolysis of different fats might serve as a basis for classification of staphylococci. Most of our results were of a negative character and we are reporting them as such.

EXPERIMENTAL PROCEDURES

Forty strains of organisms were tested, thirty-eight of staphylococci and two of *Micrococcus tetragenus*. The first nine listed were secured through the courtesy of Dr. L. A. Julianelle of St. Louis—the Type A strains being pathogenic and the Type B non-pathogenic. The source of each strain is listed in table 2.

The action of each was tested on eight different pure triglycerides: Triacetin, triproprionin, tributyrin, tri-n-valerin, tricaproin, trilaurin, trimyristin and tristearin procured from the Eastman Kodak Company, Rochester, New York.

Beef-infusion broth distributed in 200 ml. Erlenmeyer flasks was used throughout the investigation. Each fat, excepting trimyristin, was tested in duplicate. This necessitated 120 flasks—40 controls and 80 containing fat in addition to the broth. The inoculated control flasks were used as the standards with which the inoclated flasks containing fat were compared after 24 hours incubation to permit hydrolysis. For all practical purposes lipolysis of the liquid fats was complete by the end of this period. Each lot of 120 flasks was filled from the same batch of broth at an initial pH of 7.4 to 7.6. In those experiments in which liquid fats were to be tested, 50 ml. of broth were placed in each flask and the stoppered flasks autoclaved. The liquid fat emulsions were prepared in a manner similar to, though not identical with, that described by Turner (1929). A measured amount of the pure fat (usually 25 ml.) was pipetted into a clean beaker. To this was added 1 gram of gum tragacanth, then 100 ml. of hot water and the mixture beaten until a stable creamy emulsion of very fine droplets was obtained. This was then autoclaved and dispensed among the 80 flasks with a sterile calibrated syringe so that each flask received 0.25 gram of fat

giving an approximate 1:200 dilution. To each control flask was added a corresponding amount of tragacanth emulsion in water without the fat. This was found to be necessary in order to obtain good color matches in the titrations carried out, using the controls as standards.

The preparation of media containing the solid fats was somewhat more troublesome. Six and two-thirds grams of fat were added to each of three 2-liter flasks containing 1330 cc. of broth. The mixtures were then autoclaved. The fats in the hot mixtures were liquid when removed from the autoclave. As the mixtures cooled they were shaken vigorously and as the fat solidified it was dispersed evenly in very fine particles. The sterile mixtures were then dispensed in 50 ml. portions through a

TABLE 1

STRAIN	NOT SHAKEN		SHAKEN	
	cc. N/10 NaOH		cc. N/10 NaOH	
	cc.		cc.	
K	1 58	1 24	11 54	11 28
O	3 36	3 29	14 88	14 42
Gr	0 94	1 28	14 13	13 80

sterile covered funnel and burette into 80 sterile, cotton-stoppered flasks. Thus each flask received 0.25 gram of the solid fat in 50 ml. of broth making a 1:200 dilution. The purpose of this procedure is to provide a large surface for enzyme action.

Thirty flasks were inoculated at a time at intervals of an hour. All inoculations were made from 24-hour beef infusion broth cultures with the same loop. The inoculated flasks were then placed in a large incubator containing a machine devised to agitate them slowly. This was prepared by taking an ordinary Kahn laboratory shaking machine and reducing its speed to 43 complete oscillations per minute by inserting an extra pulley system. Then a double-decked platform was bolted onto the box of the shaking machine. The 120 inoculated flasks were placed on these two platforms and were agitated by the motion of the machine for 24 hours at 37 to 38°C. At the end of this

TABLE 2

C.C. 0.1 N NaOH required to titrate test flasks to the same acidity as the controls

STRAIN	TYPE OR SOURCE	TRIACETIN		TRIPROPRIONIN		TRIBUTYRIN		TRI-N-VALERIN		TRICAPROIN		TRILAURIN		TRIMYRISTIN	TRISTEARIN		HEMOLYSIS, 24 HOURS	PIGMENT, 24 HOURS
K	Type A	5 87	5 27	5 40	7 66	12 62	13 18	4 78	5 89	2 33	1 56	2 86	2 65	4 09	3.24	3 02	+++	++
C	Type A	4 48	4 49	13 58	12 73	15 87	16 99	5 92	5 51	4 39	6 36	1 76	2 47	1 75	2.99	3 64	++	+++
B_2A	Type A	5.23	5.51	13 04	12.52	16.03	16 98	3.69	5.54	5.39	5 19	2 31	2 55	2.83	0 47	0.00	0	++
13	Type A	5 77	6 67	14 83	14 22	19 05	19 03	13 04	13 42	3 04	3 45	4 92	4 73	2 52	3 38	3 62	+++	++
P	Type A	5 53	5 56	14 02	13 90	15 18	15 09	7 34	8 01	6 76	4 04	3 38	2 89	2 23	2 53	2 81	+++	+++
MX-2	Type B	6 23	5 98	18 23	17 03	18 11	17.55	10 24	10 04	8 35	8 30	5.26	4 54	3 38	3 77	3 15	+++	+
D_1	Type B	6 96	7 35	11 67	10 73	3 00	2 93	1 67	2 10	2 17	2.10	5 82		4 80	1.58	2 21	0	0
MX-31	Type B	7 86	7.62	16 07	15 63	17 73	18 91	10.49	12 11	8 40	8 29	5 02	7 38	2 40	3.12	3 67	+++	+
M-11	Type B	5 62	5 68	16 61	15 63	14 58	15 33	7 32	9 88	3 60	4 10	1 58	1 99	3 54	0.81	0.42	0	++
G-	Osteomyelitis	5 65	5 82	10 29	9 77	13 22	14 49	7 68	9.28	6 12	7.16	5.18	5 58	3 06	3 56	3 61	+++	++
O	Abscess	3 76	3.71	5.85	6 49	9 24	11 79	5 62	6 36	5 10	5 24	3 20	2 63	2 91	1.78	2 06	+++	+++
O_1	Abscess	5 77	5 49	12.23	12.56	13 58	15 04	6 05	8 01	2 09	2 17	4 08	2 78	2 52	1 87	1 78	++	+++
H	Infected scalp laceration	5 14	4 88	10.93	10 09	12 69	13 22	6 82	8 89	5 93	5 42	2 33	2 24	2 78	2 12	2 85	+++	+++
6A	Osteomyelitis	4 62	4 17	10 03	9 47	12.78	12 39	6 69	6 77	5 71	5 88	3 39	2 92	2 21	2 52	2 37	+	++
4J	Boils—arthritis	6 46	6 22	11 23	11 58	15 71	15 13	8 72	9 20	7 11	7 93	4 68	5 30	1 47	1 90	1 56	+++	++
Mc-5	Septicemia	5 32	4 92	14.17	14 32	12.22	14 69	8 36	8 12	8 88	8 85	2 97	3.20	2 77	1 51	2 12	+++	+++
W	Osteomyelitis	5 04	5 24	15 22	16 12	14 08	13 22	7 89	8 21	3 96	3 59	3 91	4 64	2 74	2 02	1 92	+++	++
B-13	Food poisoning	4 75	4 51	8 87	8 52	14 08	14 18	5 78	6 78	8 11	7 01	3 91	3 88	1 69	2 25	0 93	+++	++
G-1	Conjunctivitis	4 58	4 90	9 34	10 33	14 02	12 77	6 70	7 12	5 53	5 41	4 75	2 09	4 66	2 79	3 06	+++	++
B_1	Sty	6 38	6 25	11 14	10 80	13 19	14 55	5 63	4 81	3 76	3 07	5 02	5 08	1 59	1 34	1 42	+++	+
Gr	Osteomyelitis	5 80	6 42	12 00	12 80	16 83	16 22	7 47	6 82	7 79	6 36	5 34	3 63	2 06	2.40	1 49	+++	++
Hy	Osteomyelitis	6 64	6 61	14 88	14 80	17.69	19 65	11 00	12 18	5 18*	5 63*	4 16	5 41	2 06	2 17	2.89	+++	+
H-N	Sinusitis	5 12	5 16	11 76	11 11	11 96	12 36	6 72	6 18	4 35	8 66	2 06	2 32	1 05	1 60	2 29	++	+++
C-S	Chronic otitis media	4 96	4 99	7 87	8 04	1 42	2 33	1 21	1 84	1 58	3 35	1 37	1 39	2 29	0.68	1 05	0	0
H-d	Furunculosis	5 97	6 28	13 33	13 28	15 81	15 48	8 03	7 28	9 21	9 95	0 78	1 05	2 21	1 69	1 14	+++	+++

203	Mitral valve lesion	6 01	6 20	6.72	6 93	1 73*	1 72*	2 48*	2 54*	2 72*	2 16*	0 00*	0 00*	1 15	0 00*	0 00*	0	0
G-N	Osteomyelitis—septicemia	5 17	5 31	13 65	14 18	11.59	10 23	7 72	8 97	1.96	1 29	0 98	1 12	3 89	1 83	2 47	++	++
R-d	Osteomyelitis	5 99	6 21	11 69	10 80	14 13	12 70	7 03	6 34	4 61	5 72	5.13	4.78	4.01	3 55	4.23	+++	++
Hg	Brain abscess	6 65	6 83	9.22	9 63	13 04	15 48	8 42	8 63	5.02	5 77	3 48	4 57	2 25	3 55	3 55	++	++
Sy	Chronic osteomyelitis	4 79	5 15	12 25	12 78	14.50	14 61	6 62	7.17	4 32	4 68	2 23	2 64	1 33	2 52	2 64	++	++
HR	Otitis media	5 80	5.57	10.30	10 03	14.99	14 78	8 72	7 32	4.72	6 04	1.86	3.97	0.96	2 26	1.53	++	+
HL	Otitis media	4 90	4 99	10 28	10 02	14 52	14 58	8 57	9.00	4 73	4 94	1 94	4 06	0 57	2 82	2.44	++	+
S.S.A.	Stock *Staph. aureus*	6 08	6.12	10.49	9 87	14.67	15.10	6 77	7 22	4 22	3.12	2.74	2 67	1 94	3 03	3 85	+	++
I	Stock *Staph. aureus*	6 46	6.40	10 72	10 32	14 57	13 51	5.14	3 72	1 74	1.82	3 72	4 83	1 77	3 05		++	+
S.S.C.	Stock *S. citreus*	3 25*	3 76*	4 24*	4 27*	1 34	1 18	0 00*	0 00*	0 00*	0 00*	0 00*		0 00*	0.00*		0	**
S.Mic.	Stock *M. tetragenus*	5.88*	6 12*	4 72*	4 08*	4 67	4 86	0.66*	0.74*	0.00*	0.00*	0 00*		0.00*	1 79		+	0
Sc.S.	Staphylococcus from normal scalp	4 87	5 19	10 99	13.15	11.43	11 19	5 23	7 96	6.97	6 54	3.46		4 42	3 21		0	0
SK.S.	From normal skin	10 28	10 38	15 18	15 99	12.13	13.98	5 68	6.96	3 84	3 89	5 92		6 66	3 32		0	0
Sc.Mic.	Micrococcus—normal scalp	20 02	19 78	21 44	20 40	13 04	18 80	5 08*	4 82	1.71	1 96	1.45		4 23	2 36		0	+
2812	From blood culture	4 82	4 56	10 73	9 92	3 53	3 29	2 18	2 16	1.33	1 38	2 00		2 17	1 58		0	+

* Scant growth.

** Lemon yellow.

time they were removed for estimation of the amount of fat hydrolyzed.

The shaking machine was devised to keep the liquid fat globules and the solid fat particles from rising to the surface and clumping, thus insuring a thorough mixing of enzyme and substrate. Comparative data given in table 1 indicate the justification of this procedure. Duplicate sets using tributyrin as the substrate were inoculated and one set was agitated for 24 hours while the other was not shaken. The difference in the amount of lipolysis is outstanding.

In order to determine the effect of aeration on lipolysis a preliminary experiment was run in which aerobic and anaerobic conditions were compared. The anaerobic flasks showed only a scanty growth and no detectable lipolysis while the aerobic flasks showed good growth and extensive lipolysis. It was thus apparent that optimum lipolysis should be studied by growing the cultures aerobically and with agitation.

In estimating the fatty acid content of the incubated mixtures all flasks containing liquid fats and their control flasks were diluted with 50 ml. of distilled water. To each of the three flasks inoculated with a given strain of organism equal amounts of brom-thymol-blue were added. The control flask usually turned a bluish-green while the flasks containing fats turned yellow if much hydrolysis had occurred. The latter were then titrated with N/10NaOH to the same color as the controls. It was possible to check duplicate flasks with reasonable accuracy. In the case of the solid fat mixtures and their controls, all were diluted with 50 ml. of 95 per cent alcohol to dissolve the solid fatty acids and were then titrated after standing a few minutes. If these were diluted with distilled water no difference in color between control and fat-containing cultures was apparent. When alcohol was used as the diluting agent and the diluted mixtures were shaken and then permitted to stand a few minutes a definite difference in color developed when the indicator was added. While the estimation of the solid fatty acids was not as convenient nor could quite the same accuracy be expected as with the liquid fatty acids the method was still applicable to a

quantitative study of the relative activity of a large number of strains.

DISCUSSION OF RESULTS

Inspection of the data collected shows that with very few exceptions all the organisms studied hydrolyzed to some extent each of the fats tested. There were no striking or consistent differences between pathogens and non-pathogens. In many cases where no lipolysis could be detected it was evident that there was less growth as compared with other organisms. This was especially true of the strains 203, S.Mic. and S.S.C.

It is interesting to note that the two strains Sk-*Staphylococcus* and Sc-*Micrococcus tetragenus* (isolated from the skin and scalp respectively) had the greatest action of all the 40 strains on tri-acetin and tri-proprionin. The strain Sc-*Staphylococcus* isolated from the scalp at the same time as these two was only moderately active in this respect.

The methods employed are sufficiently accurate for an investigation of this type. It is possible to test a large number of strains in a short time obtaining data which gives a quantitative index to the lipolytic activity of the organisms under a given set of conditions and which at the same time give a basis of comparison between the strains tested. The procedure may also be employed in a qualitative sense.

A few of the less active strains show less pigmentation and some show little or no hemolysis but this is not general among the 40 strains studied.

SUMMARY

The data secured from measuring the action of 38 different strains of staphylococci and 2 strains of *Micrococcus tetragenus* on eight different pure triglycerides indicate that they exhibit considerable lipolytic activity.

While there are varying degrees of hydrolytic activity among some of the strains studied under the same conditions, there is no apparent correlation between this activity and the virulence of a given strain or its source. There was no evidence that the

lipolysis of different pure triglycerides could be used as a basis for a classification of these 40 strains of cocci.

The advantage of moderate agitation of enzyme and substrate in this type of work is apparent from the comparative data presented.

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VIABILITY OF BACTERIA IN SEA WATER[1]

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Received for publication October 23, 1936

INTRODUCTORY

It has been repeatedly found that the bacterial population in the sea is very limited. This is especially true of sea water in a natural state, although the sea floor, particularly the uppermost surface layer, may contain large numbers of bacteria. Frequently, not more than ten or a dozen bacterial cells are found in 1 cc. of sea water when it is plated out immediately upon a suitable agar or gelatin medium. Close to shore and at certain seasons of the year, this number may reach 500 to 1,000 cells, while far from land it may be as low as 1 or 2 cells per cubic centimeter of sea water. This exceptionally small number of viable cells is not due to a lack of food material in the sea water, since, when the latter is allowed to remain undisturbed in the laboratory for 24 to 72 hours, the number of bacteria capable of developing into colonies on the plate may increase to 50,000 per 1 cc. and even to 10 or 20 times that figure. An appreciable increase may take place even within 2 to 4 hours, or during the period when the sample of sea water is being brought to the laboratory.

Among the explanations suggested for the very low numbers of viable bacteria in natural waters, the following may be mentioned: (1) the presence in sea water of toxic substances which are destructive to bacteria under natural conditions (ZoBell, 1936); (2) the presence of bacteriophage in the water (Arloing *et al.*,

[1] Contribution No. 124 of the Woods Hole Oceanographic Institution; Journal Series Paper of the New Jersey Agricultural Experiment Station, and contribution from New York Medical College and Flower Hospital.

1925); (3) the adsorption of the bacteria by the sea bottom and their sedimentation (Rubentschik *et al.*, 1936); (4) the bactericidal effect of sunlight (Gaarder and Spärck, 1931); (5) the consumption of the bacteria by protozoa and other small animal organisms (Waksman and Carey, 1935); (6) the possible presence in the sea of inactive bacteria which are capable of developing only under more favorable conditions of temperature, aeration and food supply (Korinek, 1927); (7) the lack of sufficient nutriments in the water; (8) the antagonistic relations of other microörganisms; (9) inadequacy of methods commonly used for counting marine bacteria.

Although all of these suggestions may be partly justified, especially under controlled conditions, it is doubtful whether any one of them can explain the phenomena observed in the sea. Most of the investigations, upon which the above explanations rest, were carried out by introducing a pure bacterial culture or a complex natural population into a medium to which it had not been previously accustomed; an example of this is the introduction of typhoid bacteria into sewage or of sewage or river water into sea water.

It has been known since the work of Giaxa (1889) and Miquel (1891) that natural or raw water in which bacteria have grown becomes unfit as a medium for the growth of bacteria, due to the formation of certain soluble and toxic products. Although Greig-Smith (1914) has found that the toxicity is increased by boiling, and ZoBell (1936) has reported that autoclaved and Berkefeld-filtered sea water are also bactericidal, other investigators (Giaxa, 1889) have come to the conclusion that the toxic agents are destroyed by heat.

The following experiments were undertaken in order to obtain further information concerning the survival of bacteria in sea water. Either the undisturbed natural population was used or a culture of a typical marine bacterium was added to the water and its survival determined. An agar-liquefying marine organism, kept in the Culture Collection of the Woods Hole Oceanographic Institution as No. 11, was selected for this purpose; it grew rapidly and the colonies on the plate could easily be distinguished from the majority of other marine bacteria.

EXPERIMENTAL

The first experiment deals with the survival of the marine agar-liquefying bacterium in fresh sea water, in heated sea water and in artificial salt water. It was found that heating of sea water at 80°C., for 30 minutes, was sufficient for sterilization; higher temperatures and pressures were avoided to prevent formation of any toxic substances. The salt water was prepared according to the formula of Allen (1914) and sterilized as above. The sea water for this and subsequent experiments was obtained from Woods Hole Harbor, one hundred or so meters from the dock, filtered through paper and used immediately.

One hundred cubic centimeter quantities of sea water were placed in sterile 250 cc. Erlenmeyer flasks and plugged with cotton. Bacterium No. 11 was grown for 24 hours in a liquid medium containing 1 gram glucose, 1 gram peptone and 0.5 gram K_2HPO_4, in 1,000 cc. sea water. The major reason for using a medium so low in nutrients was to avoid the addition with the inoculum of any appreciable amounts of unused organic compounds to the sea water. The bacterial culture was added in 0.5 and 2.5 cc. amounts to the various flasks of water. These were incubated in the dark at room temperature (18 to 20°C.) and plated out, usually in two dilutions, on an agar medium containing the above nutrients plus 1.5 per cent agar. The plates were incubated for 48 hours and the colonies counted. This period of incubation was found to be sufficient for the development of the agar-liquefying bacterium and for a number of the common sea water bacteria.

The results presented in table 1 show that there was active bacterial multiplication in the fresh and heated sea water, as well as in the prepared salt water. The increase in bacterial numbers may have been due partly to their continued growth at the expense of the small amounts of nutrient material which still remained in the culture inoculum, and partly to a better dispersion of the cells. In the fresh sea water, the bacterial population of the water itself also began to grow rapidly. The maximum numbers were attained in 24 to 48 hours. After that period, the number of bacteria began to diminish rapidly in the

fresh sea water, reaching a minimum in 13 to 29 days. This minimum was comparable to the number of bacteria found in the uninoculated fresh sea water at the same incubation period. The plates showed few colonies of organism No. 11, but numerous colonies of the usual sea water bacteria were present. In the heated water, however, Bacterium No. 11 remained in large numbers up to 13 days and then began to decrease slowly, according to counts made on the 29th day of incubation. Between 29 and 56 days the numbers decreased rapidly. The behavior of the

TABLE 1

Survival of an agar-liquefying marine bacterium in fresh sea water, in sterile sea water, and in prepared salt water

Numbers of bacteria in 1 cc., in thousands

PERIOD OF INCUBATION	TREATMENT OF WATER								
	Untreated fresh sea water			Heated sea water			Salt water		
	Amount of culture No. 11 added								
	None	0.5 cc.	2.5 cc.	None	0 5 cc.	2.5 cc	None	0.5 cc.	2.5 cc.
Start	1 6	930	4,250	0	2,300	6,250	0	1,470	5,800
1 5 hours	2 3	2,800	19,400	0	3,780	24,800	0	1,690	15,800
4 0 hours	2	4,950	35,600	0	4,600	31,000	0	3,930	24,300
21 5 hours	29	15,100	70,000	0	5,420	23,600	0	3,260	27,400
2 days	120	9,000	56,000	0	9,500	34,000	0	2,700	29,500
4 days	79		688	0	9,150	32,500	0	2,450	27,700
7 days	100	2,140	608		7,900	34,800		2,560	31,000
13 days	52	33 5	25.5		5,300	21,200		2,980	21,500
29 days	25	2 2	29 8		2,650	9,200		700	7,400
56 days	21	39 8	63 4		88	215		105	205

bacteria added to the prepared salt water was very similar to that of the bacteria in the heated sea water.

The results of this experiment have thus brought out definitely the fact that, in fresh sea water, a destructive effect was exerted upon the cells of a marine bacterium added to it. This destruction of the bacteria was usually accompanied by the formation of a very fine coagulum throughout the water, which gradually settled to the bottom of the container.

In order to determine the survival of bacteria in Berkefeld-filtered sea water, a quantity of fresh sea water was filtered

through a Berkefeld of the W type and 100 cc. portions placed in sterile 250 cc. Erlenmeyer flasks. To separate portions of this sea water were added Bacterium No. 11 (1 cc. of a 24-hour-old culture) and sea water 24 hours old (5 cc.). The results presented in table 2 show that the marine bacteria survived readily in Berkefeld-filtered sea water and that no decided injurious effect was observed, even after 14 days incubation.

Passage through a Seitz filter modified the sea water so that the bacteria added to it were rapidly destroyed; only in the case of the larger inoculum was there a gradual adjustment of the organisms to the toxic substances in the sea water. Further

TABLE 2

Survival of marine bacteria in Berkefeld-filtered sea water

Numbers of bacteria in 1 cc., in thousands

PERIOD OF INCUBATION	NATURE OF CULTURE ADDED		
	None	Bacterium 11	Cultured sea water (24 hours old)
Start	0	3,950	0.65
1 5 hours		2,750	0 7
24 hours	0	6,650	1,300
3 days		3,650	6,050
7 days	0	2,850	1,000
14 days	0	5,860	1,060

studies brought out the fact that this destructive effect was largely due to the dissolution of some of the metal in the cup during the long filtration period that was required to collect a large quantity of sea water.

A series of experiments was now carried out for the purpose of establishing the effect of fresh sea water upon both the numbers and activities of marine bacteria. The plate method was used to estimate the number of living cells. As a measure of bacterial activity, oxygen consumption by the Winkler method seemed to be the most sensitive index and it was, therefore, used in these experiments.

Two quantities of water were prepared: (1) fresh sea water, filtered through paper; (2) sea water heated for 30 minutes at

80°C. In order to re-saturate the heated water with oxygen, the flasks containing this water were placed in a refrigerator overnight; the water was then de-saturated by placing the flasks, for a few minutes, in warm water. The oxygen content of the fresh and heated sea water was 5.43 and 5.62 per liter, respectively. Both lots of water were distributed into a series of carefully cleaned and dried 200 cc. oxygen bottles. These were inoculated with varying amounts of a 48-hour culture of Bacterium No. 11. The bottles were incubated under water in the dark and removed at various intervals for the determination of bacterial numbers and oxygen concentration.

The destructive effect of the fresh sea water upon the added bacteria was observed (table 3) within 23 hours incubation; this effect became especially striking after 3 days, when practically all the added bacteria had disappeared. In the heated sea water, however, the bacteria remained at a high level up to nine days. Although the bacteria had been reduced to a minimum in the bottles containing the fresh sea water, there was greater oxygen consumption in these bottles than in those containing the heated sea water, where the added bacteria survived in much greater numbers. The experiment was repeated, with similar results. This could lead to but one conclusion, namely, that the agents responsible for the destruction of the bacterial cells in the fresh sea water were not necessarily destructive to the processes concerned in the decomposition of organic matter in the water, as measured by oxygen consumption. Either these agents themselves consumed the oxygen in the water, or the fewer numbers of bacteria left possessed a much greater power of oxygen consumption (and, therefore, of organic matter decomposition), or a large part of the oxygen consumption was brought about by bacteria which were unable to develop on the agar plate.

In the following experiment, an attempt was made to obtain further information in regard to the relationship between the survival of bacteria in sea water and oxygen consumption, to search for the presence of other organisms in sea water which might modify bacterial activities, and to determine the changes taking place in the water during the early periods after the addi-

tion of the bacteria. All the bottles were sterilized by dry heat, for 18 hours at 60 to 65°C. Several check determinations were made, to eliminate the variability of the individual bottles, which had been frequently obtained in the previous experiments. A

TABLE 3

Survival of an agar-liquefying marine bacterium in fresh and in heated sea water

NATURE OF WATER		FRESH		HEATED	
Incubation	Inoculum added	Bacteria in 1 cc.	Oxygen consumed	Bacteria in 1 cc	Oxygen consumed
	cc.	*thousands*	*cc. per liter*	*thousands*	*cc. per liter*
3.5 hours	0				
3.5 hours	0.1		0.06	910	0.10
3.5 hours	0.3	1,650	0.09	2,250	0.16
3.5 hours	1.0	5,750	0.39	8,150	0.61
23 hours	0	8.5	0.12	0	0
23 hours	0.1	685	0.66	1,200	0.16
23 hours	0.3			2,335	0.25
23 hours	1.0	3,250	2.16	5,150	0.82
3 days	0				
3 days	0.1	26	0.75	1,490	0.40
3 days	0.3	31	1.66	2,140	0.43
3 days	1.0	6.5	4.38	10,680	2.74
5 days	0	2.8	0.39	50	0.01
5 days	0.1	4.9	0.87	980	0.40
5 days	0.3	5.5	1.74	1,850	0.70
5 days	1.0	7.0	5.16	6,390	2.89
9 days	0				
9 days	0.1	Few		440	0.52
9 days	0.3	4.5	2.40	1,350	0.88
9 days	1.0	5.5	5.43*	5,350	3.58
15 days	0				
15 days	0.1	0.9	1.17	115	0.58
15 days	0.3	1.6	2.46	1,930	1.18
15 days	1.0	2.8	5.43*	1,850	3.88

* Oxygen all used up.

24-hour culture of No. 11 was employed. The results presented in table 4 again confirmed those obtained previously.

In the case of the fresh sea water, the bacterial maximum was obtained within 24 hours, followed by a drop in the number of bacteria. Within 3 days, practically all the added bacteria had

disappeared and were replaced by the native bacterial population of the water, the numbers being of about the same magnitude in the inoculated and uninoculated sea water. The slight difference in favor of the inoculated sea water may possibly be due to the traces of nutrients added with the inoculum. On the other

TABLE 4

Growth and oxygen consumption of a marine bacterium in fresh and heated sea water

NATURE OF WATER		FRESH		HEATED	
Incubation	Inoculum added	Bacteria in 1 cc.	Oxygen consumed	Bacteria in 1 cc.	Oxygen consumed
	cc.	*thousands*	*cc. per liter*	*thousands*	*cc. per liter*
Start	0	2.7	0	0	0
Start	0.3	720	0	500	0
Start	1.0	2,050	0	2,080	0
4 hours	0	4.5	0		
4 hours	0.3	1,260	0.20	2,820	0.36
4 hours	1.0	6,700	0.72	6,500	0.90
9 hours	0	9	0.11	0	0
9 hours	0.3	1,590	0.36	2,990	0.52
9 hours	1.0	6,300	1.01	9,000	1.12
24 hours	0	45	0.23		
24 hours	0.3	3,100	0.95	3,420	0.64
24 hours	1.0	7,100	2.75	12,200	1.41
2 days	0	11	0.45	0.04	0
2 days	0.3	173	1.32	3,800	0.72
2 days	1.0	370	4.16	9,900	1.59
3 days	0	10.5	0.53	62	0.12
3 days	0.3	18*	1.59	3,720	0.78
3 days	1.0	14*	4.71	9,500	1.71
6 days	0	2.4	0.71	2	0.15
6 days	0.3	5.4*	2.09	2,970	1.14
6 days	1.0	18.2*	5.33†	8,800	2.97

* No. 11 practically all disappeared.

† Oxygen all used up.

hand, in the case of the heated sea water, where maximum numbers of bacteria were also obtained in 24 hours, the numbers remained very high even after 6 days incubation, actually 4 to 6 times as high as at the start.

The oxygen consumption in this experiment was similar to that previously found. In the fresh sea water, there was a gradual

disappearance of the oxygen, the rate of its consumption depending on the amount of inoculum added. The greater consumption of the oxygen was due both to the presence of larger numbers of bacterial cells and to greater concentration of the unconsumed nutrients. In practically all cases, the ratio for oxygen consumption in the bottles receiving 0.3 and 1.0 cc. of the inoculum was similar to the ratio of the bacterial numbers in the original inocula. However, after 24 hours incubation, the increasing oxygen consumption was quite independent of the numbers of bacteria since the latter decreased rapidly, while the former, being cumulative, continued to increase. In the heated sea water, the bacterial numbers remained very high even after 6 days incubation, the total number per bottle with 1 cc. inoculum being 1,760,000,000 or four times as great as the number of bacteria originally introduced. The oxygen consumption was still considerably less than that in the fresh sea water.

The water from several of the bottles was centrifuged at the end of the incubation periods and the resulting sediments examined microscopically for the presence of protozoa and other animals. This examination revealed the fact that the heated water contained a large number of bacteria in addition to some dead diatomaceous material. The fresh sea water, on the other hand, contained many protozoa, a number of copepods (12 living forms having been counted in one bottle) and various other lower animal forms.

One may draw from these experiments two broad conclusions, namely, (1) that sea water contains an agent or agents destructive to marine bacteria, and (2) this agent does not interfere with the processes of marine organic matter decomposition, as measured by oxygen consumption. These seemingly contradictory conclusions are possible only by assuming that the agents which are largely responsible for the destruction of the bacteria form a part of the system which is responsible for the decomposition of the organic matter in the sea water. This explanation can help in understanding the reasons for the low bacterial population in natural sea water; the bacterial population is largely held down through the activities of the numerous animals inhabiting the sea.

DISCUSSION

The abundance of bacteria in sea water is controlled by a number of factors, chief among which are (1) the amount and nature of available food material; (2) the rate of bacterial multiplication, leading to the formation of substances injurious to bacterial development; (3) the nature and abundance of the animal population, ranging from minute protozoa to larger forms; (4) the environmental conditions, as exemplified by temperature, and aeration or stagnation of water.

It has been amply demonstrated that extensive bacterial multiplication takes place in sea water, either as a result of (*a*) adding fresh organic matter in the form of algal or of animal residues; (*b*) keeping the water in an undisturbed condition; (*c*) warming the water. Rapid bacterial growth is sooner or later followed by a drop in numbers; the extent of the drop and the equilibrium which is finally established depend on the nature of the stimulus and on the amount of residual organic matter in the water.

The drop in bacterial numbers is greatly delayed and its rate is much slower, when the bacteria are added to sterile sea water instead of to the same water in a fresh state. The bacterial products and the exhaustion of available nutrients cannot, therefore, be alone responsible for the low numbers of bacteria which remain in fresh water in a living and active state. It is believed that another factor, namely the animal population, largely the protozoa and other members of the nannoplankton in the water, is also responsible for bringing about the reduction in the numbers of bacteria. This assumption is substantiated by two facts, first, the multiplication of these organisms in fresh water in which bacterial development has taken place, and secondly, the greater oxygen consumption in the fresh sea water as compared with the sterile sea water, in spite of the greater numbers of bacteria in the latter.

Consumption of oxygen is a measure of the respiratory processes of living systems. When light is excluded, especially in the case of water containing only a limited number of diatoms, the process

of oxygen consumption is carried out by the bacteria and the animal population. The reduction in bacterial numbers accompanied by the growth of animal forms can only mean that the latter are partly responsible for both the destruction of the bacteria and the consumption of the oxygen.

The limited numbers of bacteria under natural conditions in the sea can thus be explained by a state of equilibrium between bacterial multiplication and bacterial destruction. A change in this equilibrium is brought about by a change in food supply, environmental conditions and modification of agents unfavorable to bacteria, whether these are simple organic substances resulting from bacterial metabolism, bacteriolytic substances, bacteriophagic agents, or organisms feeding directly upon the bacteria.

SUMMARY

1. A study has been made of the survival of a marine bacterium added to fresh sea water, to sea water sterilized by heat or by filtration through a Berkefeld filter, and to prepared salt water.

2. The fresh sea water exerted a marked destructive effect upon the cells of the marine bacterium added; this effect followed an initial rise in numbers. Sterilized sea water and prepared salt water had no destructive effect upon the bacteria.

3. The destructive effect of fresh sea water upon the bacteria was not accompanied by a similar effect upon the rate of decomposition of the organic matter in the water, as measured by oxygen consumption.

4. It is suggested that the activities of the animal population of the sea, especially the nannoplankton, can explain, at least partly, both the destruction of the bacteria and the stimulation of the processes of organic matter decomposition.

5. These facts are used as a basis for explaining the low numbers of bacteria usually found in natural sea water.

The authors are indebted to Dr. M. Butler for assistance in making the oxygen determinations.

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ADSORPTION OF BACTERIA BY INERT PARTICULATE REAGENTS

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Received for publication October 26, 1936

Persons suffering from intestinal intolerance of carbohydrates have been treated, with more or less promising results, with kaolin given by mouth (Althausen, Gunnison, Marshall and Shipman, 1935). Kaolin, Fuller's earth, charcoal, aluminum hydroxide, and other substances inconstant in physical and chemical composition have been similarly used in cholera, the dysenteries, ulcerative colitis, and numerous other intestinal disorders (Swalm, 1934; McRobert, 1934; Mills, 1935). As an explanation of a part of the mechanism whereby such substances proved clinically beneficial the hypothesis has been advanced that the intestinal flora is altered due to a somewhat selective adsorption of bacteria.

The present report concerns experiments designed to indicate the degree to which apparent adsorption of bacteria on finely dispersed adsorbing mediums may occur under certain experimental conditions. It is obvious that conditions in the intestinal tract vary in many respects and between wide limits. Factors which influence adsorption; namely, the degree of dispersion of the reagents, the variable chemical composition of such reagents, the hydrogen ion concentration, the surface shape of dispersed particles, the fluidity of suspensions, the degree to which mechanical contact may be established, and other factors represent variables in the intestinal tract to which must be added the more complicated anatomical and pathological variables. These variables are far too numerous to control in test tube experimentation, yet the data indicate in some instances such marked adsorption and in other instances, notably with *Clostridium*

welchii, such definite failure to adsorb that the results may be worthy of record. Furthermore, it becomes evident that test tube experiments furnish an insufficient background for an hypothesis ascribing to simple adsorption the beneficial symptomatic results in patients.

It was concluded by Eisenberg (1918) that Gram-positive organisms were readily adsorbed but that Gram-negative organisms were not. A change in intestinal flora from a proteolytic type to an aciduric type, accompanied by a virtual elimination of *C. welchii* from the stools was reported by Braafladt (1923). Experiments *in vitro* by the latter author led him to conclude that kaolin carried down with it large numbers of bacteria when mixed with them and kept in motion for several hours. Whether such broad generalizations concerning purely physical adsorption are justified is problematical.

EXPERIMENTAL

Reagents used to adsorb were kaolin (Baker, washed and ignited), Lloyd's reagent (Lilly), barium sulphate (Mallinkrodt, U. S. P. for X-ray diagnosis), calcium carbonate (Merck, precipitated U. S. P. X.), colloidal aluminum hydroxide (Kolloyd Laboratories) and bone charcoal. The reagents were sterilized by dry heat.

The majority of tests was performed with *Escherichia coli*, *Clostridium welchii* and *Lactobacillus acidophilus*, each at pH 7.2 and at pH 5.6. Some additional tests were made with *Staphylococcus aureus*, *Sarcina lutea*, and *Bacillus subtilis*.

In the first series of experiments the technic described by Braafladt (1923) was used. One gram of adsorbent was mixed with 10 cc. of beef extract broth, buffered at pH 7.2 or at pH 5.6. To this 0.1 cc. of a 24-hour broth culture of bacteria was added. The mixture was incubated at 37°C. for 3 hours, with gentle shaking at 5-minute intervals. The adsorbent was removed by gentle centrifugalization, and a control tube without adsorbent was similarly handled. The pH value, determined at the end of each experiment, changed from 0.2 to 0.4. Organisms present in the supernatant fluid were counted. When pertinent, the pro-

cedure used in standard water analysis was followed. For *L. acidophilus* whey agar plates were counted in 48 hours, and for *C. welchii* serial dilutions were prepared in liver agar shake tubes.

Similar tests were carried out with suspensions of fecal material. One gram of feces was suspended in 10 cc. of broth containing 1 gram of adsorbent. Control tubes were set up without adsorbent. After incubation, shaking and centrifugalization, counts were made from the supernatant fluid according to the method used in water analysis.

For a more extended series of tests the procedure used by Eisenberg (1918) was modified. One-tenth of a gram of adsorbent was added to 5 cc. of a suspension of bacteria in buffered saline. This suspension was prepared by emulsification of the growth of a 24-hour agar slant to a point of bare turbidity. Control tubes contained no adsorbent. The suspensions were gently agitated during a 15-minute period. Following centrifugalization organisms in the supernatant fluids were counted as in the first series.

Variable factors tested included the number of organisms, the concentration of adsorbent, the period of contact, and the degree of shaking. Numerous tests in which centrifugalization was omitted were made to check possible toxicity of the reagents.

RESULTS

Although the Braafladt method permitted multiplication of aerobic organisms during the course of the experiment, there was no significant difference between the results obtained in the two series. For this reason the results of an extended series are combined in a single table (table 1). In this table the proportion of organisms removed with centrifugalization of the adsorbent is expressed as a ratio of the concentration of organisms in the control supernatant fluids to the concentration of organisms in the corresponding "adsorbed" tubes.

No perceptible toxicity was shown by the reagents used in these experiments for the organisms tested. The adsorbents exerted no germicidal action nor did they inhibit growth. Braafladt found virtually no toxicity with kaolin.

Repeated experiments, which were attempted in each case not

less than 3 nor more than 11 times, gave an index of proper evaluation of the results. The variation was marked and implied a delicate balance of uncontrolled factors. Thus, on a conservative statistical basis, any apparent adsorption which did not exceed tenfold (90 per cent reduction) should not be regarded as experimentally significant, although the variation permits conjectures as to its causes. The more marked apparent adsorptions are illustrated by reductions in bacterial concentration to from 1/40 to 1/1600 of the original concentration, essentially a different order of magnitude from the range of 1/2 to 1/10 reduction.

TABLE 1

Summary of 234 "adsorption" experiments

ORGANISM	APPROXIMATE pH	RATIO—BACTERIA PER CC. CONTROL : "ADSORBENT"					
		Kaolin	Lloyd's	Charcoal	$CaCO_3$	$Al(OH)_3$	$BaSO_4$
E. coli	7.2	2	1.5	5	1 5	2	2
	5.6	5	3	8	*18*	2	6
C. welchii	7 2	1	1	1	1.5	1	1
	5 6	1	1	1	1 5	1	1
L. acidophilus	7.2	2	1	*1,600*	1	6	2
	5.6	7	1	*380*	1	*16*	*39*
Staphylococcus	7 2	*640*		*630*			
	5 6	*720*		*920*			
Sarcina	7 2	*600*					
B. subtilis	7 2	*700*					

In experiments with stool suspensions the variations in results were naturally more marked, since such tests introduced many variable factors. No reductions of significant magnitude were obtained.

If adsorption occurred at all it seemed to take place within the first 15 minutes of contact. Table 2 illustrates with a typical experiment the similarity in results secured following 15-minute and 3-hour exposures.

Similarly the concentration of organisms in suspension seemed to have no significant effect. In table 3 is given an experiment

in which the bacterial concentration was varied with 3 different adsorbents. The figures again represent the ratio of organisms per cubic centimeter in the supernatant fluid of control tubes to the organisms per cubic centimeter in the supernatant fluid of adsorbed tubes; i.e., the magnitude of reduction in bacterial concentration.

TABLE 2

Failure of increased time to influence degree of "adsorption"

PERIOD OF CONTACT BETWEEN ADSORBENT AND BACTERIA	NUMBER OF E. COLI PER CC. OF SUPERNATANT FLUID		RATIO—BACTERIA PER CC. CONTROL "ADSORBED"
	Kaolin, 1 gram	Control	
15 minutes	965,000	2,250,000	2.3
3 hours	195,000,000	450,000,000	2.3

TABLE 3

Failure of variation in number of organisms to influence degree of "adsorption"

NUMBER OF ORGANISMS PRESENT—E. COLI	RATIO—BACTERIA PER CC. CONTROL "ADSORBED"		
	Kaolin, 1 gram	Charcoal, 1 gram	$BaSO_4$, 1 gram
20,000	2.0	1.3	1.0
2,000,000	2.3	3.7	2.9
17,000,000	3.1	1.1	1.0
45,000,000	1.0	1.1	1.0
183,000,000	1.0	1.4	1.6
360,000,000	1.0	5.3	1.3
460,000,000	1.2	5.3	1.2

The use of 5 or 10 times as much adsorbent in one series as in the other did not significantly alter the results, nor did variation of the violence of agitation have any marked effect.

As seen in table 1, adsorption was more marked at pH 5.6 than at pH 7.2 with one exception; viz., with *L. acidophilus* and charcoal.

Attempts to demonstrate microscopically an affinity between adsorbents and bacteria met with failure. The variation in size and shape of particles of adsorbents was microscopically marked.

DISCUSSION

Strictly interpreted, marked apparent adsorption of the intestinal organisms used, *Escherichia coli*, *C. welchii*, and *L. acidophilus* was exhibited with only one combination, viz., charcoal and *L. acidophilus*, especially in neutral or alkaline suspension. No noticeable effect in any single experiment was noted with *C. welchii*, an organism around which much controversy has centered in the clinical use of some of these reagents although the clinical significance of this organism is debatable (Gunnison, Althausen and Marshall, 1936). In clearly acid suspensions it is possible that a noteworthy removal of *E. coli* by calcium carbonate, and of *L. acidophilus* by aluminum hydroxide and by barium sulphate might occur. Adsorption near the isoelectric point of the organism should theoretically be more marked.

Kaolin appeared to remove staphylococci, a sarcina, and *B. subtilis* in a marked degree. In fact, in an experiment, not recorded because incompletely checked, a reduction of *Sarcina* exceeding 13,000 times was observed at pH 5.6. Staphylococci were also notably reduced by charcoal.

Although results tabulated above agree with those of Eisenberg in so far as organisms used by him were tested, his conclusion that Gram-positive organisms are readily adsorbed was not uniformly confirmed. It is probable that factors not associated with dye affinity are also operative. Most of the Gram-positive organisms tested by Eisenberg formed chains or capsules, whereas the Gram-negative organisms did not, a point which suggests that the correlation he noted might have been based on purely mechanical removal of what chanced to be Gram-positive bacteria. Eisenberg removed his adsorbents by filtration, a procedure which introduced another factor.

Recent studies by the Soviet workers (Rubentschik, Roisin and Bieljansky, 1936), published after the completion of the present study, are essentially supported by it. They also raised objection to Eisenberg's generalization. The chief difference between the results of these authors and the results herein reported rests on their observation that an increase in the number of organisms

or in the concentration of adsorbent increased the degree of adsorption.

Braafladt concluded that kaolin carried down a large number of bacteria, a conclusion based on a three-fold reduction in concentration of *E. coli* suspensions. A comparable reduction was secured in these experiments but comparison with other organisms suspended with other adsorbents suggest an opposite conclusion. If Braafladt's observations on the removal of *C. welchii* from the intestinal tract by kaolin were correct, the results would seem to have had an explanation in purely physiologic phenomena, or in mechanical factors such as an alteration of the physical state of the intestinal contents. This organism not only failed to be adsorbed under any conditions tested *in vitro*, but studies of human stools failed to reveal any decrease in *C. welchii*. (Gunnison, Althausen and Marshall, 1936). It seems logical, to suppose that the adsorption of toxins or even of enzymes, in connection with more abstruse physiologic changes, such as coating of the intestinal mucosa, is more likely to account for reported clinical improvements than the removal of bacteria by adsorption as is so often assumed. Most of the experiments which have led illogically to the hypothesis of bacterial adsorption have been hopelessly dissimilar to conditions of the intestinal tract.

It is frankly recognized that the data secured are not practical in a clinical sense nor are they exacting enough for the colloid chemist. There are too few dependent variables for the clinician, and too many for the chemist. Experiments with the staphylococci particularly, however, indicate that what appears to be adsorption may be demonstrated by the technical procedures used.

That the observed phenomenon is true adsorption, directly dependent on the electric charge of reagent surfaces, on the pH, on the opportunity for "collision," on the surface tension relationships between the cell membrane and the particle surface to be filmed and similar factors is doubted. Different adsorbents did not appear to "adsorb" in proportion to their charges. In fact, the species of bacteria seemed more important than either the pH or the adsorbent. It is a popular approach in bacterio-

logic problems to consider bacterial suspensions as similar to colloidal suspensions or emulsions. This concept is legitimate only in the degree to which the surface: volume relationship in a bacterial suspension enters into any given phenomenon to an extent similar to that of a true colloid. In this instance, however, bacteria are of an order of magnitude not greatly different from the particles of kaolin or other reagents. Both bodies are relatively too large to come within the realm of colloidal phenomena. If, as may happen in the few extreme examples observed, bacteria do come in contact with inert particles, attempt to wet them, and become fixed around such particles perhaps by electrical attraction it would still tax the imagination to call the phenomenon adsorption. The argument advanced by Kramer (1928) and others that the adsorption of toxins, bacteriophage, viruses, tuberculin, adrenalin, Congo red, *et al.* has a parallel in bacterial adsorption seem invalid, although there may be some correlation between the factors involved. Bacteria presumably are composed of colloidal systems, but the whole cell is much too large and too complex to view it as a colloidal particle. One is dealing with the wrong order of magnitude to think in terms of adsorption, and the experimental observations made during the course of the present study and in similar studies suggest a need for a more definitely physiologic inquiry by the clinician and for a more direct mechanical inquiry by the bacteriologist. The recent study from Odessa (Rubentschik, Roisin and Bieljansky, 1936) implied strong objections to the adsorption concept in spite of the descriptive convenience of the term.

CONCLUSIONS

The apparent adsorption of *Escherichia coli*, *Clostridium welchii* and *Lactobacillus acidophilus* by particulate kaolin, Lloyd's reagent, calcium carbonate, aluminum hydroxide and barium sulphate *in vitro* was not marked. Charcoal was effective in removing *Lactobacillus acidophilus* from suspension, but did not remove significantly *Escherichia coli* and *Clostridium welchii.*

Using organisms not primarily found in the intestinal tract, *Staphylococcus aureus*, *Sarcina lutea* and *Bacillus subtilis* were

tested with kaolin which removed each of these organisms; and charcoal, tried with staphylococci only, removed this species.

Eisenberg's hypothesis that Gram-positive organisms are more easily removed from suspensions than Gram-negative organisms was not substantiated. The assumption that kaolin removes large numbers of organisms from suspensions, and therefore acts similarly in the alteration of intestinal flora was not supported. It is believed that other factors are responsible for intestinal changes following the use of inert particulate agents.

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A SIMPLE METHOD FOR PRESERVING BACTERIAL CULTURES BY FREEZING AND DRYING[1]

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Received for publication October 27, 1936

Numerous studies on colony forms and on antigenic structure and virulence of bacteria have demonstrated conclusively that these microörganisms are constantly undergoing significant variation when they are repeatedly subcultured on artificial media or subjected to animal passage. The desirability of maintaining type-culture collections, as well as cultures for use in any laboratory, in the state in which the bacteria were originally isolated is obvious; while the cost of artificial media and the labor involved in frequent subculturing are additional incentives for establishing suitable techniques for satisfactorily preserving bacteria. Drying while in the frozen state has been shown to accomplish this end; the viability, virulence, immunological and biochemical characteristics of bacteria and certain other infectious agents are all maintained. For example: we have recently found, viable and still possessing their group specific substance, cultures of hemolytic streptococci preserved in this way in 1916–17. Many other strains of hemolytic streptococci have been similarly stored in this laboratory within the past twelve years during the course of studies of their antigenic components and colony forms (Lancefield, 1933); these have been recovered at intervals and have consistently maintained the characteristics noted at the time of their original preservation.[2] Pneumococci similarly preserved have kept their type specificity. Rake has found that certain strains of pneumococci, having a very labile

[1] Presented at the Second International Congress for Microbiology, London, July 25 to August 1, 1936.

[2] I am indebted to Dr. R. C. Lancefield for these studies.

capacity to invade the blood stream of mice through the nasal mucosa, quickly lose this capacity unless they are preserved by freezing and drying. He (1935) has further observed that such delicate bacteria as meningococci have maintained their original virulence and specific carbohydrate content for at least 20 months when preserved in the same manner.

Elser, Thomas and Steffen (1935) have had similar experiences with many strains of bacteria. Filterable viruses may also be preserved if infected tissues or exudates are frozen and then dried; for instance, the yellow fever virus has been kept for at least five years in the Laboratories of the International Health Board (Sawyer and Bauer).

In most of the work mentioned above the bacteria were frozen in a salt-ice mixture; but there is reason to believe that protoplasm may be less injured when more quickly frozen at −76°C. Turner (1936) has recently maintained the viability and virulence of *Treponema pallidum* at this low temperature for 4 to 6 months, while, on the other hand, several years ago I was unable to demonstrate any viability in similar treponemas in rabbit chancres frozen at −6° to −10°C. In the frozen food industry it has been shown that much smaller crystals are formed in fish quickly frozen at −76°C. than in that more slowly frozen at higher temperatures (Fitzgerald), and it seems probable that the smaller crystals are less harmful to the frozen cell.

Many bacteria will live in a simple dried state for considerable lengths of time, with gradual reduction in viability, a fact that has led to the method of preserving cultures by drying them, without freezing, on filter paper or other fibrous or granular material. When bacteria are suspended in a colloid, such as serum or blood, and then dried without preliminary freezing, they may retain their original characteristics for many months. Recently Stillman has observed this phenomenon with pneumococci dried in defibrinated rabbit blood, where control cultures dried simply in broth succumbed in a few weeks. Dried infected exudates and tissues, such as spleens, are also useful agents for preserving some bacteria.

A combination of freezing and drying for the preservation of biological products was suggested years ago by Shackell (1909)

and used shortly afterwards for keeping rabies virus (Harris and Shackell, 1911), and bacteria (Hammer, 1911). We improved the technique a few years later and described the method and results of its application in 1921 (Swift). Elser has accomplished the same purpose with a different form of apparatus which he and his collaborators have recently described (Elser, Thomas and Steffen, 1935). Their article also contains a good review of the literature on this subject. Later, Sawyer and his co-workers (Sawyer, Lloyd and Kitchen, 1929) devised an apparatus which is automatic in its action; and still more recently Flosdorf and Mudd (1935) have perfected their so-called "lyophile" apparatus which has many excellent points.

The principle involved in all techniques is the removal of the water from the material while it is still frozen; thus a harmful concentration of salts and other substances is obviated. Two methods are employed for removing the water from the frozen substances, both of which are carried out *in vacuo*. In one the water vapor is absorbed by a chemical desiccant; in the other it is removed by low temperature evaporation and condensation—a modified distillation. The latter is used in the apparatus of Elser, Thomas and Steffen and in that of Flosdorf and Mudd. These methods are specially applicable in the desiccation of immune sera and other biological products, since large quantities of water can be readily removed, and they also have the additional advantage that the special containers may be sealed with the contents under a high vacuum. The apparatus is, however, more expensive to purchase and to use than the more simple equipment here described, in which a chemical desiccant is employed. The apparatus described by Sawyer and his co-workers also makes use of a chemical desiccant. Its chief advantage is the certainty that the material will remain frozen until dried; but it is rather cumbersome and too expensive for many laboratories.

APPARATUS

The method originally described by us (Swift, 1921) and the modifications more recently developed can be carried out with equipment usually available. All methods require a good vacuum pump such as the Hyvac.

Desiccator. A desiccator with *well ground joints* and a *good stop cock* is essential. It contains glycerol, a manometer and a dish of P_2O_5. The ground glass surfaces are coated with a mixture, one part of paraffin, melting point 56°C., and 6 parts of vaseline. The small U-shaped mercury manometer is fastened to the inner side of the desiccator with adhesive plaster. Glycerol, with all traces of water removed, is placed in the bottom of the desiccator

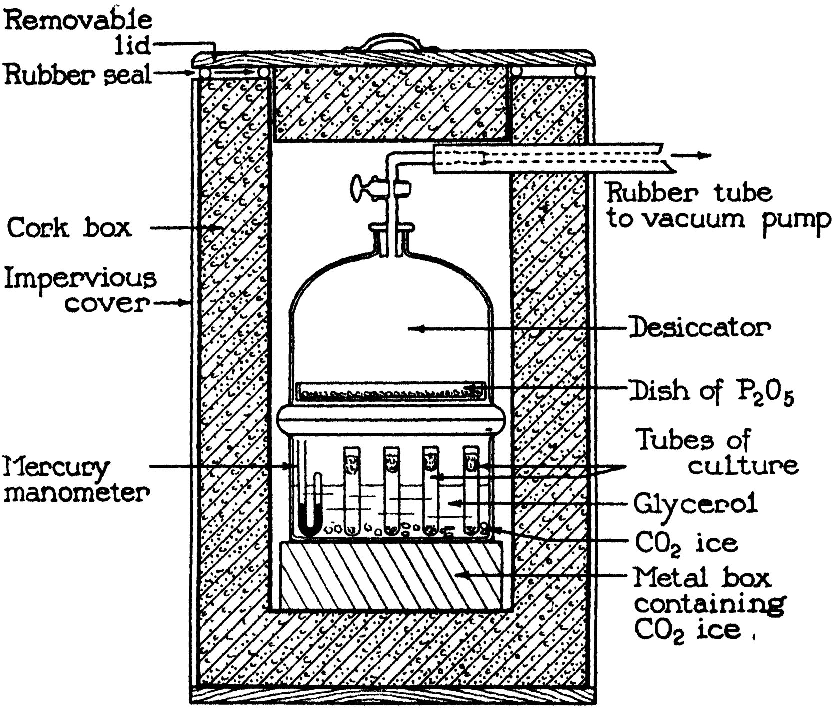

FIG. 1. DIAGRAM OF CORK REFRIGERATING BOX CONTAINING DESICCATOR EMPLOYED FOR FREEZING CULTURES WITH CO_2 ICE, AND DRYING WHILE IN THE FROZEN STATE

to a depth of 2 to 3 cm., and this glycerol is kept free of water by maintaining a vacuum in the desiccator when it is not in use. A large flat dish, such as half of a large Petri dish, is held in readiness for the desiccant P_2O_5, which is used as described below. This desiccant has proven more satisfactory than sulfuric acid or calcium chloride because of its more rapid action, as well as its greater hygroscopic power.

Refrigerating box. Since CO_2 ice has become readily available a simplified method of preserving cultures—called Method B—has been devised. For this a simple refrigerating box, that can be easily moved, has many advantages. Ours is built of 3-inch cork board which is glued together with a special cement.[3] The inside diameter is slightly larger than the desiccator, and the depth is sufficient to hold the latter and a metal box as shown in figure 1. The cork-board, insulating the removable top, is glued to a pine lid, and the bottom is also reinforced with a pine board. The sides of the box are entirely covered with closely woven canvas which is painted with several coats of impervious paint. The seal under the lid consists of two rows of rubber tubing. The lid is held in place simply by gravity, for a tight mechanical seal must be avoided on account of the pressure of CO_2 gas developed. A hole in the upper part of the refrigerating box permits the passage of heavy rubber pressure tubing from the pump to the desiccator. In the bottom of the cork box is placed a flat-topped sheet metal box containing CO_2 ice. The desiccator rests on this box.

TECHNIQUE OF METHOD B[4]

The refrigerator box is cooled by placing, from 200 to 400 grams of solid CO_2, in the metal container. The exact amount must be learned by experience with the conditions in each laboratory, for only enough is needed to keep the inside of the apparatus slightly below 0°C. until desiccation is complete. An excess will maintain a temperature so low that the process of drying is retarded.

Young, actively growing, broth cultures of the bacteria are centrifuged, the supernatant fluid is discarded, and the sediment is resuspended in broth or serum to about 1/25th of the original volume of culture.[5] In the case of bacteria which must be grown

[3] I wish to thank the Armstrong Cork Company for furnishing the cork board and cement and for useful suggestions in making the box.

[4] I am specially indebted to our technician, Stephen Nalesnyk, for many useful suggestions in the development of Method B.

[5] While in all of our work broth has been employed as a suspending medium, it seems that serum may be better in the case of extremely delicate bacteria or viruses. This is suggested by the experience of Stillman with the simple drying

on solid media, the surface growth is scraped off and suspended in broth or serum. The bacterial suspensions are distributed in small tubes (7 x 120 mm.) in 0.1 cc. amounts; and the cotton plugs are pushed down until their upper surface is about 5 mm. below the tops of the tubes. CO_2 ice is crushed and scattered over the top of the glycerol in the desiccator; the amount, varying between 300 and 500 grams, depends upon the quantity of material to be frozen. This CO_2 ice is quickly pushed to the bottom of the glycerol with the tubes of concentrated culture. The glycerol soon solidifies. The simultaneous introduction of the CO_2 and tubes results in the cultures being rapidly reduced to a temperature of about −76°C. The top is firmly pressed on the lower part of the desiccator so that a tight seal is formed between the ground glass surfaces which have been previously covered with a fresh coating of the paraffin and vaseline mixture. Sealing is facilitated by having the desiccator connected with the running vacuum pump; in fact, it is necessary to maintain a partial vacuum, because the rapid evolution of gas from the CO_2 ice may blow the top off the desiccator unless the gas is rapidly removed. The desiccator is placed in the cooled refrigerating box with as little interruption of the pumping as possible, and after an inspection of all connections the lid is placed on the cork box. Obviously, while the CO_2 is evaporating only a partial vacuum is possible, but during this period some water is probably being removed from the frozen cultures. The high vacuum required for satisfactory desiccation will not be attained until some time after the CO_2 is entirely evaporated; hence it is well to keep the pump running until an hour or more after this evaporation is completed. For this reason it is advisable not to put too much CO_2 ice in the desiccator, only enough to insure rapid freezing of the cultures, and maintenance of the frozen state until the cultures are dry. The amounts will depend upon the size of the

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of pneumococci. Furthermore, Rivers and Ward (1935) have noted that cultures of vaccine virus remain viable much longer in the dried state following freezing when they are previously mixed with a colloid such as 3 per cent gum acacia; and Dochez and his coworkers (Dochez, Mills, and Kneeland, 1936) have observed the same with cultures of virus of the common cold.

apparatus, the initial temperature of the glycerol, and the amount of material to be treated. It is convenient to have a good vacuum gauge, connected by means of a three-way stop cock situated between the desiccator and the pump, in order to determine when a sufficiently high vacuum has been reached. A vacuum of less than 1 or 2 mm. of mercury is necessary for satisfactory results, and with a good set-up with a Hyvac pump a pressure of 30 to 50 microns is obtained; this is desirable, since, in general, the better the vacuum, the more satisfactory are the ultimate results. About an hour after this high vacuum has been attained the stop cock is closed. With proper equipment and a good set-up the contents of the tubes should be dried at least by the following morning.

Before opening the desiccator the air should be allowed to enter very slowly. While it has been our custom to allow natural atmospheric air to enter the desiccator, it would probably be better to pass it over calcium chloride or some other non-volatile desiccant, for in this way one could be more certain of a minimal amount of water vapor coming in contact with the dried culture.

Sealing: With very delicate material, as for instance some of the viruses, it is probably better to use the Flosdorf-Mudd apparatus which permits sealing with the contents of the tubes under a high vacuum. If this is not available and the desiccator method is used the material should be placed in longer tubes; then, as recommended by Elser, Thomas and Steffen (1935), the tubes can be constricted in a blow pipe flame just below the level of the cotton; they may then be connected to a vacuum pump, without removing the cotton plugs, and secondarily evacuated, after which they can be closed by fusing the constricted portion of the tubes.

With most bacteria, however, we have found a wax seal applied at atmospheric pressure more suitable on account of convenience both in closing and opening the tubes. Greater safety is attained because contamination of the cultures by an inrush of air is prevented, and there is no danger of a spread of the dried bacteria into the surrounding atmosphere. Consequently, we have employed the following technique: The tubes are removed from the

desiccator, freed of glycerol, and placed in rows in suitable racks. *Without delay* melted wax is allowed to run into the space above the cotton. The wax must be *very hot* in order to adhere well to the glass and make a perfect seal. A micro-bunsen burner is very satisfactory for melting the wax and heating the top of the tubes so that the melted wax drops directly into the space above the cotton plug. Air bubbles in the wax are removed by secondarily heating the tubes as they are rotated. Following this, a second application of wax is made. We have used successively paraffin wax, sealing wax, and finally Picein, as seals. The first has the disadvantage of not making a permanently tight seal; consequently the plug moves, especially in hot weather (see tube 6, figure 2); sealing wax has been better, but is liable to break if the tubes are placed in the refrigerator or other cold environment; and although Picein is less liable to break than sealing wax because it is less brittle, we have observed, even with this substance, that rapid chilling may induce thin cracks between the Picein seal and the wall of the tube. For this reason we have kept the dried cultures in file boxes at room temperature rather than in the cold, and have found them to remain unaltered in their virulence, serological and other biological characteristics, for many years (probably indefinitely if the seals remain intact).[6]

The tubes are conveniently opened as follows: A piece of iron wire (we use a straightened-out paper clip) is heated red hot and pushed through the wax, then allowed to cool. When the wax is cool, the upper end of the tube is heated gently in the pilot flame of a bunsen burner so that the outer part of the wax plug is melted while the core remains solid; the plug is then easily withdrawn by means of the wire previously inserted. The cultures are recovered by placing suitable fluid media directly in the tube. In those instances where the organisms grow best on solid medium the dried material may be fished out and inoculated on suitable media.

[6] It is probable that a cement, such as Dipar Liquid Sealing Cement, recommended by Flosdorf and Mudd (1935) painted over the surface of the wax plugs and the top of the glass tubes, would make a more permanent seal resistant to the cracking that comes from cooling the tubes. Such a final sealing is especially to be recommended when the tubes are to be shipped long distances or exposed to winter temperatures in which they may be overcooled.

Properly dried material has a characteristic appearance which varies according to the substance used for suspending the bacteria. With broth the dried material will have a white or light yellow

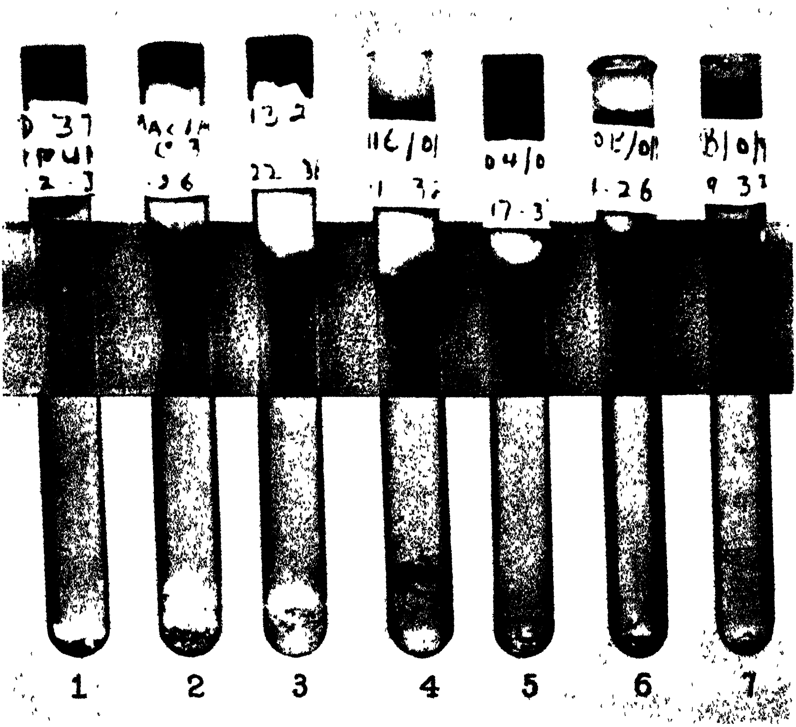

FIG. 2. APPEARANCE OF FROZEN AND DRIED CULTURES UNDER VARIOUS CONDITIONS

1, properly dried culture originally suspended in serum. 2 and 3, properly dried cultures originally suspended in broth. 4, culture which was properly dried but which is becoming gummy due to breaking of paraffin wax seal. 5 and 6, cultures which have become gummy due to breaking of seals. Note how the seal in tube 6 has been pushed out during the hot weather. 7, culture probably melted before being completely dried. Note films of dried material high up on the wall of the tube. Cultures in tubes 5, 6 and 7, not viable; and that in 4 of doubtful viability.

color and a loose frothy consistency somewhat like dried shaving lather (tubes 2 and 3, fig. 2). If serum was used as a suspending medium, the material will remain in the shape of a solid compact

cap and have a firmer appearance (tube 1, fig. 2). If the culture has melted before being completely dried, it will appear to have boiled, with a thin film high up on the side of the container and a yellow, thick, gummy film adhering tightly to the bottom of the tube (tube 7, fig. 2). Moreover, if properly dried material takes up much moisture from the air it will assume the same gummy aspect (tubes 4, 5 and 6, fig. 2). Such cultures will usually not be viable.

ORIGINAL METHOD—METHOD A

Where CO_2 ice is not available, the original method (Swift, 1921) which we employed for many years, can be easily carried out. The tubes of concentrated culture are immersed in a salt-ice mixture until the contents are well frozen. They are then transferred to the desiccator to be dried, with the precaution of carefully wiping all salt water from the outside of the tubes before they are immersed in the glycerol. The desiccator is also previously immersed in a salt ice mixture which chills the glycerol to about $-6°C$. After the tubes are all in place, a dish of P_2O_5 is placed above them, the desiccator is sealed with the paraffin-vaseline mixture and the air evacuated with a Hyvac pump. A good vacuum is essential for this method also. The desiccator is kept cold by immersion in a salt-ice mixture, or in a brine bath connected with a refrigerating system. Suitable modifications in the ordinary electrical refrigerators found in many laboratories, might also be applicable to the maintenance of sub-zero C. temperatures. Again, it should be emphasized that the cultures must be kept frozen until they are completely dried, which is usually accomplished overnight. Melting is more liable to take place in Method A than in Method B; and, as has been mentioned, it is possible that freezing at the very low temperature of $-76°C$., as is done in Method B, has advantages.

After the cultures are dry the remainder of the procedure is exactly the same as that described for Method B. Adhesive plaster marked with water-proof India ink makes a satisfactory label; for it is to be noted that good permanent labels are especially desirable on cultures which are to be stored for an indefinite period.

SUMMARY

1. Bacteria maintain their original cultural, immunological and biochemical characteristics and their virulence for many years at room temperature when they are completely dried while in a frozen state, provided the cultures so dried are well sealed to keep out water vapor.

2. Two methods are described for freezing and drying material. Both require the maintenance of a high vacuum until the cultures are dry and both employ P_2O_5 as a desiccant. In Method A the cooling system is a salt-ice mixture which is used both for freezing the cultures and for reducing the glycerol in the desiccator to a sub-zero temperature, and some special provision must be made for keeping the cultures frozen until desiccation is complete. In Method B the cultures are frozen in the desiccator by immersion in glycerol to which solid CO_2 is added; and suitably low temperatures are maintained by placing the desiccator in a cork insulated box, containing a small amount of CO_2 ice. Either Picein or sealing wax make satisfactory seals provided the tubes are never subjected to ice-box temperatures.

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THE GRAM-NEGATIVE BACTEROIDES OF THE INTESTINE

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Received for publication November 3, 1936

In 1933 Eggerth and Gagnon published a paper entitled "The Bacteroides of Human Feces." The results presented were illuminating in that they aided materially in the opening of a new field in intestinal bacteriology and broadened existing knowledge concerning non-sporulating anaerobes.

Eggerth and Gagnon state that in 91 per cent of a series of 60 stools of adults the predominating organisms were obligate anaerobes of the genus *Bacteroides*. They isolated and made a taxonomic study of Gram-negative *Bacteroides*.

Eggerth and Gagnon's observations naturally lead one to assume that these organisms must play a rôle of prime importance in the intestine, and that they constitute valuable material for further intensive investigation. No further stimulus was required for the present writers to enter this new field.

The following were points of outstanding interest: (1) the relative numbers of bacteroides in human feces; (2) the proportion of Gram-negative to Gram-positive non-sporulating anaerobes; (3) a comprehensive morphological, cultural and serological study, and (4) the adoption of a definite system of classification of the isolated strains.

Over 40 stools of human adults were cultured aerobically and anaerobically. A total of 87 anaerobic strains[2] was isolated. To this number were added 22 strains received from Eggerth. These 109 organisms were studied systematically, together with

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seven strains of *Lactobacillus bifidus*, four of *Lactobacillus acidophilus* and 1 of *Lactobacillus bulgaricus*. Of the 109 different so-called *Bacteroides* strains, 36 were Gram-positive and 73 Gram-negative. The Gram-negative organisms will be dealt with in this paper.

TECHNIC

The methods and media used by Eggerth and Gagnon were adopted, with a few slight changes. Quantitative determinations were not attempted. The common procedure consisted in making saline suspensions of the feces having an approximate nephelometer reading of 5.0 (MacFarland). With this as dilution 1, 9 further dilutions were made, the dilution being doubled each time. Platings were made on blood agar from dilutions 3 to 10, inclusive, and duplicate plates incubated aerobically and anaerobically.

The basal medium was the one employed by Eggerth and Gagnon, namely beef infusion agar containing 1.5 per cent agar, 1 per cent peptone, and 0.4 per cent disodium phosphate; the final pH was 7.6 to 7.8. At the time of the pouring of the plates 7 per cent defibrinated sterile cows' blood and 0.15 per cent sterile glucose solution were added. In accordance with the observations of Eggerth and Gagnon, an acid reaction was found to be unfavorable to the initial growth of these forms; however, after the organisms were obtained in pure culture, a slight acidity proved to be the most favorable for nearly all strains.

The blood agar plates were incubated usually at 37.5°C. for periods varying from 1 week to 10 days. Anaerobic methods which depend entirely on evacuation were very unsatisfactory due to the drying out of the plates. A procedure was employed (Weiss and Spaulding, 1937) which involves evacuation and filling the jar with tank hydrogen, and the use of palladinized asbestos for the complete removal of the free oxygen. This method proved very satisfactory.

In the isolation of pure cultures, inoculum from selected colonies was streaked on new blood agar plates, and the plates incubated under the usual anaerobic conditions. The process of

re-plating was continued until there was every indication of the plates containing the organism in pure form. This was followed by spore tests. All organisms which were non-spore-forming obligate anaerobic rods were inoculated into egg-meat and held for further study.

Eggerth and Gagnon kept most of their stock cultures of Gram-negative *Bacteroides* strains in brain medium. As this is rather specialized and expensive tissue, and as our laboratory carries the ordinary egg-meat medium regularly, the latter was tried out. All of the strains of Gram-negative and Gram-positive non-sporulating anaerobes appeared to grow well and retain their viability in it; it was adopted, therefore, as the standard stock medium.

The *Bacteroides* group studied here is non-proteolytic. On opening anaerobic jars containing them, a strong offensive odor pervades the laboratory. However, at no time were there any signs of digestion in the egg-meat tubes except when they were contaminated with anaerobic spore-forming bacteria. To avoid contamination from wet plugs, all tubes and plates were covered with paper towels. The members of this group remained alive for 3 months or longer in the egg-meat tubes, at both room and ice box temperatures.

Blood agar and egg-meat are not entirely satisfactory media. The ideal medium would be one that contains all of the required nutrient substances and is sufficiently clear to permit an unobscured view of the bacterial growth. This goal is being sought by another member of this laboratory who is engaged in a study of the group from angles somewhat different from those discussed here.

All special media, such as carbohydrates, milk, gelatin, tryptophane broth, lead acetate and nitrate broth were made according to the directions of Eggerth and Gagnon.

RELATIVE NUMBERS OF BACTEROIDES IN STOOLS

Our results bear out those of Eggerth and Gagnon, in general. The majority of fecal samples examined revealed the predominating organisms to be members of the *Bacteroides* group. In

many instances *Bacteroides* colonies occurred on the agar plates in apparently pure form, in the higher dilutions of feces. As a rule the best isolations were obtained between the fifth and sixth dilutions; or, between the second and the fifth after the last tube showing visible turbidity. At no time were spore-forming anaerobes observed in large enough numbers to appear in any of the higher dilutions. *Escherichia coli* was the only organism which survived the elimination process, and then only occasionally.

It is but natural to assume that any organism which appears in the intestine in as high concentrations as these non-sporulating anaerobes are found to do, must play some important rôle in the intestine. Such an assumption alone should be a strong stimulus to prosecute intensive search into their nature, food requirements, activities and exact relations to other bacteria and to the host.

Eggerth and Gagnon included both the Gram-negative and Gram-positive non-sporulating anaerobes of the intestine in the so-called genus, *Bacteroides*, although they are apparently open-minded. Because the Gram stain is generally regarded as significant in any classification scheme the present report is devoted to the Gram-negative group, with the intention of making the Gram-positive division the subject of a future paper. This paper then deals with a study of 73 Gram-negative *Bacteroides* strains, 15 of which were obtained from Eggerth and Gagnon, and the remainder isolated in our laboratory from the feces of presumably normal human adults.

What constitutes the *Bacteroides* group or genus? Is it an entity in itself, or is it a group of organisms which is too inclusive, and should be split up into definite and distinct genera? The Gram-negative representatives may be set apart for the moment from all other known organisms, except perhaps the fusiform group, by the fact that they are definitely non-spore-forming obligate anaerobes which do not retain Gram violet stain. There have been numerous references in the literature to Gram-negative non-spore-forming anaerobic rods, but until we can obtain a better description of them, all such forms must be classified under

the head of *Bacteroides* or *Fusobacterium.* The *Bacteroides* genus is not limited to the intestinal tract; in fact, organisms resembling this group have been isolated from other sources as, for example, the respiratory passages (present authors), and inflammatory lesions (Henthorne, Thompson and Beaver, 1936).

In differentiating the *Bacteroides* from the *Fusobacterium* group some difficulty is encountered. This confusion is more apparent than real and is due largely to faulty description. For the most part, the fusiform group is more rapid in its growth, and the cells are longer than those of the known *Bacteroides;* furthermore, they often possess tapering ends, and distinct granules. The fusiform group of bacteria requires highly specialized media. There are, however, some so-called borderline types which in certain respects resemble both the *Bacteroides* and *Fusobacterium* genera.

MORPHOLOGY

The Gram-negative *Bacteroides* vary rather markedly in their morphology, according to their age and the type of medium upon which they are grown. They are small rods, as a rule. When grown on blood agar, the cells vary from 0.3 to 0.7μ in thickness, and from 0.5 to 3.0μ in length. There are, of course, exceptions to the rule. Some *Bacteroides* members may even become filamentous at times. All of the strains studied here were non-motile.

As will be shown later, morphological characteristics agree fairly well with each other and supply a logical basis for dividing these organisms into several well-defined sub-groups or species.

The majority of the strains appear as solid-staining rods after 4 days incubation at 37°C. on blood agar. Some may show bipolar staining, and many appear quite granular. Most of the strains are ovoid in cell form, and some become quite coccoid, so that it is often difficult to identify them as rods.

Growths on glucose agar usually reveal a distorted morphology. Growth in broth of any kind leads to material departure from the so-called normal cell form and stimulates the development of long filamentous or large oval forms which contain granules and vacuoles.

The cells may occur single or in pairs; often they are grouped in clusters. Chain formation may be observed.

COLONY MORPHOLOGY

The colonies on blood agar plates may vary from pin points up to 3 to 4 mm. in diameter. The average size may be given as 1 to 3 mm.

There are several types of colonies, the most common appearing soft, grayish, elevated and opaque, and varying from 1 to 3 mm. in diameter. A second type of colony is grayish but rather transparent, while still another is the pin-point type of colony, inoculum from which usually refuses to grow on glucose agar.

The colonies vary in consistency. Some are moist, others quite dry in appearance; some are mucoid, and others distinctly soft. Nearly all of the colonies have smooth edges; a few strains produced an undulating edge. Most colonies are round and elevated or "dew drop" like in appearance; some may assume peculiar shapes such as rising to a point or peak. Some strains exhibit marked hemolysis on blood agar, but this reaction is not observed often.

BIOCHEMICAL PROPERTIES

All of the strains grow fairly well and retain their viability in egg-meat, which is used as the regular stock culture medium. Nearly all develop in infusion, or better still, in glucose infusion broth. The type of growth is usually diffuse. Acid is formed in the glucose broth, depressing the pH to around 5.0 to 5.4.

A majority of the strains acidify and coagulate litmus milk. Most of them liquefy gelatin after 30 days at 37.5°C. Indol is formed by a few strains. Lead acetate is frequently blackened, but at no time has reduction of nitrates to nitrites been noted.

FERMENTATION PROPERTIES

Twenty fermentable substances were employed in these tests. The results of Eggerth and Gagnon were confirmed to a fair degree. The monosaccharides were utilized almost universally, as was lactose. Almost all of the other test substances were

attacked in varying degrees by the different strains. Sorbitol and glycerol were utilized only occasionally, and dulcitol and inositol were never fermented. Our results were at variance with those of Eggerth and Gagnon, in a few instances, especially when small amounts of serum were added to the fermentation medium.

Eggerth and Gagnon used the fermentation tests as a basis for classification. After evaluating our results, and after noting the differences between them and those of Eggerth and Gagnon, we were forced to conclude that a classification based largely on such reactions is illogical. It must be admitted that the fermentation properties of individual strains vary over different periods of time and under even slightly different environmental conditions. Indeed, on studying our results and attempting to correlate them with the serological grouping no evidence was found in favor of any classification based on fermentation reactions. For this important reason the fermentation results are not presented here in full in spite of the fact that the group as a whole is active, fermentatively, especially in some media as, for example, deep agar tubes.

PATHOGENICITY

Various suspensions of the different *Bacteroides* cultures were injected into white mice, guinea pigs and rabbits by the intraperitoneal and intravenous routes. In two instances the animals died after the injections, but subsequent injections of the same strains did not confirm the original results. From the combined results in these experiments we may safely conclude that the organisms employed are non-pathogenic for mice, guinea pigs and rabbits. Whether these organisms may assume a pathogenic or otherwise harmful (or beneficial) rôle in the intestine, their natural habitat, is at best only problematical.

SEROLOGICAL PROPERTIES

Eggerth and Gagnon (1933) stated that they had not succeeded in their attempts to develop agglutinogenic antisera for the *Bacteroides* group.

Assuming at this stage of the investigation that serology offered

our main, if not only, hope of classification, we undertook to produce several antisera. The procedure involved the use of large and heavy inoculums freshly prepared from blood agar plates. The bacterial cells were, after 5 or 6 washings, injected into rabbits. Each of the animals received at least 16 injections, with the result that 10 antisera of a fairly satisfactory titer were developed. Most of the sera had agglutination titers of 1:320, and several as high as 1:10,240. Moreover, the sera did not appear to be strain-specific, and promised to offer opportunity for a logical classification of the Gram-negative members of the genus.

Antigens were prepared with washed cells from blood agar plates. The agglutination tubes were set up in the usual manner and examined after 24 hours holding at 37°C. Additional readings were made after further holding for 24 hours, and after 48 hours at room temperature. One antiserum showed a pro-zone reaction.

PROPOSED CLASSIFICATION

Rather than postulating, as Eggerth and Gagnon suggested, 18 species in this group, and these based almost wholly on fermentation reactions, we propose a simpler classification founded largely on morphological and serological data. The wisdom of adopting such a plan, and its permanency, can be determined only after much more extensive taxonomic research in this field. The classification proposed by Bergey (1934) and based on reactions in litmus milk in no way harmonizes with the data obtained by us and, in our judgment, rests on faulty premises.

Proposed classification based chiefly on morphology and agglutination reactions

All anaerobic, non-spore-forming non-motile Gram-negative rods.

Group I. (The most common division of the *Bacteroides* genus)

Type strain and proposed name: *Bacteroides vulgatus* (Eggerth and Gagnon).

Cell morphology: Ovoid, solid-staining rods, occasionally bipolar-staining, appearing either single or in pairs. Sometimes grouped. Average size 0.5 to 0.7 × 1 to 1.5μ.

Colonies: On blood agar usually elevated, grayish and opaque with smooth edges. Average diameter 1 to 3 mm. Glucose agar colonies usually smaller, but growth fairly good.

Infusion broth: Growth diffuse and usually heavy; pH of glucose broth culture between 4.8 and 5.4, most strains about 5.0.

Litmus milk: Usually acidified, without coagulation.

Indol: Variable, most strains negative.

Gelatin: Variable, most strains liquefying.

Lead acetate: Positive.

Fermentations: Variable.

Nitrates not reduced.

Non-pathogenic for mice, guinea pigs and rabbits.

Serologically quite well grouped, although there are a few exceptions.

Optimum temperature: 37.5 C.

Habitat: Intestinal tract.

Of the strains named and described by Eggerth and Gagnon, the following fall into group I:

Bacteroides vulgatus
Bacteroides insolitus
Bacteroides incommunis
Bacteroides convexus

While the following organisms were not available for study, they probably fall into the same division (group I):

Bacteroides thetaiotaomicron
Bacteroides uniformis
Bacteroides distasonis
Bacteroides tumidus
Bacteroides ovatus
Bacteroides siccus

Group II. (Another prominent division of the genus *Bacteroides*)

Type strain and proposed name: *Bacteroides varius* (Eggerth and Gagnon).

Cell morphology: Granular staining oval bacillus, 0.5 × 0.5 to 1μ. Organisms usually appear in clusters, or grouped in pairs, occasionally short chains.

Colonies: On blood agar usually gray, elevated, entire, 2 to 3 mm. in diameter. They vary from opaque or slightly opaque to trans-

parent. Pin point colonies may appear. This group does not grow as well as group I on glucose agar.

Infusion broth: Growth in plain and glucose broth usually heavy and diffuse. Final pH in glucose broth around 4.8.

Litmus milk: Usually acidified and coagulated.

Indol: Positive.

Gelatin: Variable, mostly liquefying.

Lead acetate: Positive.

Fermentations: Variable

Nitrates not reduced.

Non-pathogenic for mice, guinea pigs and rabbits.

Anaerobic, non-spore forming, non-motile, Gram-negative rods.

Serologically quite well grouped.

Optimum temperature: 37.5°C.

Habitat: Intestinal tract.

Of the strains named and described by Eggerth and Gagnon, the following belong in this group:

Bacteroides gulosus
Bacteroides varius

The group should probably also include:

Bacteroides coagulans

Group III. (An uncommon member of the genus)

Type strain and proposed name: *Bacteroides uncatus* (Eggerth and Gagnon). This group seems to be set apart from all of the others by agglutination, morphology and cultural reactions.

Cell morphology: Slender pointed bacilli; curved—hooked forms present. Vary from 0.5μ to distinct filamentous form. Average size 1 to 3μ. They appear to be closely related to the *Fusiformis* genus, morphologically.

Colonies: On blood agar colonies are usually very small or pin point. Glucose agar generally shows no growth.

Infusion broth: Growth is diffuse, but not nearly as heavy as that of the other groups. The pH of glucose broth varies between 5.6 and 6.0, never falling below 5.6.

Litmus milk: Variable.

Indol: Negative.

Gelatin: Positive (liquefying).

Lead acetate: Negative. The only organisms which do not brown lead acetate fall in group III.

Nitrate: Not reduced.

Non-pathogenic for mice, guinea pigs and rabbits.

Serologically well grouped.

Optimum temperature: 37.5°C.

Habitat: Intestinal tract.

Of the strains of Eggerth and Gagnon, the following belong in this group (III):

Bacteroides uncatus
Bacteroides vescus
Bacteroides exiguus

Group IV

In many classification schemes there is a so-called waste-basket group. Group IV constitutes such a group here; it is made up of organisms which agree morphologically and culturally with some other group, but fail to be related to the same groups serologically.

Of the strains described by Eggerth and Gagnon there are two which should fall into group II morphologically and culturally, but which show no serological relationship to this group, or to any other group, or to each other. These strains are:

Bacteroides variabilis
Bacteroides inaequalis

DISCUSSION

As stated before, the above classification is based primarily on agglutination properties, and secondarily on morphology. The differentiation of the groups is, on the whole, quite clear-cut; however, there are certain exceptions. Several of the strains studied belong definitely to group I, morphologically and serologically; nevertheless, they cross-agglutinate with group II serum, although not in as high dilutions as with their own. Group III is distinct both morphologically and serologically from the others. Group IV, as has been stated, shows no serological relationship to any other group, but does resemble group II morphologically.

It would be possible to further subdivide groups I and II on

the basis of certain cultural and biochemical characteristics, especially the reactions in gelatin and the production of indol. We do not feel justified, however, in making such a division, in view of the fact that the serological results would not support it.

Detailed descriptions of the organisms are not given here, since they would require more than the permissible amount of Journal space.

CONCLUSION

The Gram-negative members of the *Bacteroides* genus are the predominant organisms in the intestine of most human adults. This observation is in agreement with those of Eggerth and Gagnon.

The Gram-positive members of the group are quite distinct from the Gram-negative, and should be considered apart from the latter.

Our morphological and cultural studies are, on the whole, in agreement with those of Eggerth and Gagnon. We do not believe, however, that a final classification should be based on them alone.

A classification of the Gram-negative organisms into 4 groups based primarily on serology, and secondarily on morphology, is proposed here. Such a classification is in the direction of greater simplicity.

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THE RELATION OF BACILLUS SIAMENSIS AND SIMILAR PATHOGENIC SPORE-FORMING BACTERIA TO BACILLUS CEREUS

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Received for publication November 10, 1936

Reports of pathogenic motile aerobic bacilli are common in the literature, as witnessed by the publications of Charrin and DeNittis (1897), Legros and Lecène (1901), Bainbridge (1903), Stregulina (1905), Ponder (1911), Wilamowski (1912), Senge (1913), Lindberg (1916), Fiori (1924), Sweany and Pinner (1925), Fleischner (1926), Bais (1927), Grierson (1928), Gilbert and Coleman (1929) and others. Many of these workers identified their isolations as "pathogenic subtilis" or as "motile, anthrax-like" bacilli; others employed various specific designations that have not received subsequent general recognition. Grierson (1928) has used "*Bacillus anthracoides*" of Bainbridge and others to include motile, experimentally pathogenic bacilli recovered by him. Bergey (1934), on the other hand, has placed "*Bacillus anthracoides*" as synonymous with *Bacillus megatherium*, and Ford (1927) has suggested its identity with *Bacillus cereus*. Recently, Siribaed (1935) has described a new pathogenic variety of *Bacillus subtilis*, which he named *Bacillus siamensis*.

The attempt to identify a number of pathogenic, aerobic bacilli isolated in the course of other work prompted us to make a study of this group. It is the purpose of this paper to present the results of this study and to attempt to place these organisms in a correct taxonomic position.

[1] The author is indebted to N. R. Smith for valuable suggestions offered in the completion of this work and in the preparation of the manuscript.

SOURCE OF CULTURES

Our attention was first attracted to this group when a *Bacillus* quickly fatal to guinea pigs after subcutaneous inoculation was recovered from a blood culture taken in a pyrexia diagnosed as scarlet fever. Later, nine strains of similar bacilli were isolated from air and soil, indicating that the original strain recovered from blood culture was probably a chance contaminant of no etiologic significance. About this time Siribaed (1935) published his description of *B. siamensis.* Its reported properties suggested possible identity with our cultures. We obtained a culture of *B. siamensis* and found it to be identical with our isolations. The question then arose as to the proper classification of this group. Since the majority of our strains had been readily isolated from air and soil, a comparative study with other "hay bacilli" was deemed necessary before confirming *B. simensis* as the species recovered in this laboratory.

This comparison embraced, altogether, the following 24 cultures:

1. *B. siamensis,* Siribaed's strain, received from Dr. Ivan C. Hall.
2. A pathogenic bacillus isolated from contaminated blood culture.

3–11. Bacilli similar to the above strains, and variously isolated from air, dust and soil.

12. *B. subtilis,* A.T.C.C. No. 102 (The American Type Culture Collection strains employed in this study were obtained from N. R. Smith).
13. *B. subtilis,* N. R. Smith No. 103.
14. *B. subtilis,* N. R. Smith No. 104.
15. *B. cereus,* A.T.C.C. No. 21.
16. *B. cereus,* N. R. Smith No. 156.
17. *B. cereus,* Smith and Clark No. 249.
18. *B. megatherium,* A.T.C.C. No. 71.
19. *B. megatherium,* Smith's R strain from B. megatherium above.
20. *B. megatherium,* N. R. Smith No. 157.
21. *B. vulgatus,* A.T.C.C. No. 123.

22. *B. mesentericus*, A.T.C.C. No. 76.
23. *B. mycoides*, A.T.C.C. No. 80.
24. *B. malabarensis*, N. R. Smith No. 150.

The identification of cultures 12 to 14 as *B. subtilis* is in agreement with the definition of this species recently recommended by Breed and St. John-Brooks (1935).

MORPHOLOGIC, CULTURAL AND PATHOGENIC PROPERTIES

Our pathogenic cultures were indistinguishable from *B. cereus* in morphologic and cultural characteristics. Observations on cultures 1 to 11 and cultures 15 to 17 were as follows:

Morphology. Large Gram-positive rods, 1.0 to 1.2μ by 3.0 to 6.0μ in size; granular staining reactions common. Early abundant sporulation; spores were centrally or paracentrally located and swelled the rods slightly when mature.

Motility. Active motility. Numerous peritrichous flagella were demonstrated on strain 2.

Colony form. Discrete agar colonies either round or irregular, with edges slightly wavy or distinctly irregular and branching. Rough strains were developed from several different smooth strains. All colonies possessed a dull, ground-glass, anthrax-like appearance; with 10× or 20× magnifications, minute parallel curls were to be seen on the surfaces of colonies.

Blood agar. Marked hemolysis.

Beef broth. White, friable surface pellicles, which sank readily when the culture tubes were handled.

Gelatin stab. Rapid saccate liquefaction.

Nitrate broth. Nitrates were reduced to nitrites.

Milk. A slight or variable coagulation followed by rapid peptonization.

Blood serum. Partial liquefaction.

Potato. Growth thick, moist, spreading, greyish-white, becoming yellowish with aging.

Fermentations. An acid fermentation of glucose, levulose, galactose, maltose, starch and glycerol was readily demonstrated in a medium employing ammonium phosphate as the source of nitrogen; in beef peptone broth with added carbohydrate, acid production was frequently masked by proteolytic cleavages. There was strain variation in the

rapidity with which acid was produced from sucrose. There was no fermentation of xylose, arabinose, mannose, lactose, mannitol or inulin in either synthetic or peptone broths.

Pathogenicity. Culture 2, when first recovered from blood culture in 1934, was fatal to guinea pigs in the amount of 1.0 cc. of 24-hour glucose-broth culture given subcutaneously. Within 6 to 12 hours after injection, guinea pigs became restless and rough-haired, and weakness of the hind quarters soon became evident. Such weakness led progressively to general prostration, respiratory difficulty, convulsions and death. Necropsy, performed immediately, showed slight hemorrhagic congestion and edema at the site of inoculation and marked congestion of the abdominal viscera. Smears from the surface of the liver and from the heart's blood showed large Gram-positive bacilli; cultures of the heart's blood yielded the inoculated organism.

During the two weeks following the original series of animal injections, a number of guinea pigs were killed with individual doses of from 1.0 to 2.0 cc. of broth cultures. However, later tests with strain 2 showed appreciable loss in virulence. After one month of subculturing on nutrient media, 2.0 cc. of broth culture did not, but 4.0 cc. did kill guinea pigs. After three months, 4.0 cc. were not fatal, but one-half the surface growth on a blood agar slant after incubation at 37°C. for 24 hours sufficed to kill a guinea pig.

Culture 1 (*B. siamensis*) was reported by Siribaed to be pathogenic to guinea pigs in amounts from 0.5 to 2.0 cc. of 24-hour broth culture. We first tested this strain for virulence in November, 1935, and found that amounts of 3.0 cc. or less were not lethal, although one-half the surface growth on a blood agar slant readily killed a guinea pig. Two weeks later, after several subcultures on blood agar, 3.0 cc. of a 24-hour broth culture sufficed to kill a guinea pig. This positive result was checked several times within the following week. Several months later, after rather intermittent cultivation, similar amounts did not kill guinea pigs.

Cultures 3, 5, 6, 8, 9 and 10, immediately after their isolation from air and soil, were lethal to guinea pigs when 4.0 cc. or more of their broth cultures were given intraperitoneally. Four cubic

centimeters of broth culture of either strain 4 or 7 failed to kill guinea pigs, but the total surface growth of either at 24 hours on a blood agar slant was fatal.

In our first tests of *B. cereus* for virulence, cultures 15 and 16 failed to kill guinea pigs either when the total surface growth at 24 hours on plain agar slant or when 4.0 cc. of glucose broth culture were injected. Such results were in accord with the common opinion that *B. cereus* is not pathogenic. These two *B. cereus* strains were then subcultured rapidly for a few generations on blood agar slants, and tested again for pathogenicity. For each, the surface growth at 24 hours on one blood agar slant was then sufficient to kill a guinea pig over night. Subsequently, 4.0 cc. of a 24-hour glucose broth culture of *B. cereus* No. 15 was found fatal to guinea pigs, although an equivalent amount of No. 16 did not kill.

DIFFERENTIATION OF THE "PATHOGENIC" STRAINS FROM OTHER BACILLI

Certain morphologic and cultural characteristics of *B. subtilis* distinguish it from *B. cereus* and the related pathogenic strains. A few such characteristics are mentioned:

Morphology. Slender Gram-positive rods, 0.7 to 0.9μ by 1.7 to 3.0μ in size.

Colony form. Thin, irregular, grey-white membranous colonies, with the mat or ground-glass appearance noted for *B. cereus* absent.

Beef broth. Membranous pellicle which did not fragment or sink readily.

Potato. Growth wrinkled and dry; at first greyish-white, but becoming pink in color on aging.

Fermentations. In ammonium phosphate medium, acid was produced in xylose, arabinose and mannitol as well as in all those sugars fermented by the *B. cereus* group of strains.

Pathogenicity. *B. subtilis* strains were consistently non-pathogenic. Even after rapid subculture on blood agar, the entire surface growth produced on two blood agar slants during 24 hours of incubation at 37°C. could be injected intraperitoneally with no appreciable pathogenic effects.

B. megatherium was readily distinguished from *B. cereus* and the related strains 1 to 11. Certain distinguishing characteristics of *B. megatherium* are mentioned:

Morphology. Very thick Gram-positive rods, 1.5 to 2.0μ by 2.5 to 4.0μ in size.

Colony form. Smooth, soft, cream-colored colonies, with surfaces possessing a uniform glistening rather than a dull appearance.

Gelatin stab. Slow crateriform to stratiform liquefaction.

Beef broth. Turbid, with no distinct surface pellicle.

Nitrates. Not reduced.

Fermentations. In ammonium phosphate medium, acid was produced in xylose, arabinose, mannose, raffinose, lactose, mannitol, dextrin, salicin and inulin as well as in glucose, levulose, galactose, maltose, sucrose, starch and glycerol.

Pathogenicity. As with *B. subtilis,* no pathogenicity was demonstrated for strains of *B. megatherium.*

B. cereus and the related pathogenic strains were readily distinguished from other common spore-forming aerobes included in this comparative study. The arborescent, rhizoid colonies of *B. mycoides* on nutrient agar, the wrinkled, folded, dry growths of *B. vulgatus* and *B. mesentericus* on potato and other solid media, and the thick, glistening, acid-producing growth of *B. malabarensis* on lactose media are mentioned as exemplary distinctions.

DISCUSSION

The complete morphologic and cultural harmony that obtained between "*B. siamensis*" or similar "pathogenic" cultures isolated here and *B. cereus* indicate that the "pathogens" are *B. cereus.* In view of the variable pathogenicity of "*B. siamensis*" and the feeble experimental pathogenicity demonstrated for *B. cereus,* it would seem inadvisable to attempt any differentiation on the basis of virulence.

No justification was encountered for considering the "pathogens" as varieties of other aerobic spore-formers. The appreci-

ably smaller cells formed by *B. subtilis*, its more membranous colony, its dry wrinkled growth on potato and its fermentation of mannitol prevented us from considering "*B. siamensis*" as a strain variation of *B. subtilis*. Again, the appreciably thicker cells of *B. megatherium*, its smooth colonies, its growth in broth without pellicle formation, its failure to reduce nitrates and its fermentation of lactose readily distinguished this species from *B. cereus*.

Ford has suggested that the feebly pathogenic "*B. anthracoides*" of some workers was probably *B. cereus*. We are in agreement with his opinion. "*B. anthracoides*," as recently described by Grierson (1928), produced anthrax-like colonies on agar, fermented glycerol but not mannitol nor lactose, and was in other respects similar to the observations given above for *B. cereus*. It is also probable that some of the "anthrax-like" or "pathogenic subtilis" bacilli of earlier workers were merely strains of *B. cereus*. That such "pathogens" were frequently encountered is not surprising. Our "pathogens" were isolated almost at will from air and soil. *B. cereus* was the species most frequently encountered by Lawrence and Ford (1916) in studies of aerobic spore-formers in milk and water from the Baltimore vicinity. It would appear desirable, in order to avoid further confusing reports of new, pathogenic, motile bacilli, that further new species should not be described until actual comparisons had been made with cultures of *B. cereus*; thus further duplications of this common species might be avoided. The significance of our work is not in the demonstration of pathogenic, motile, aerobic bacilli, since this has been done repeatedly by others during the last four decades, but rather in the positive identification of such bacilli with *B. cereus*, one of the most common of the aerobic spore-formers in soil. This series of observations, begun in a laboratory dealing primarily with pathogenic microörganisms and completed in a laboratory of soil microbiology, is a striking case of the break-down of that specificity which has relegated certain species to the pathogenic field and others to the saprophytic.

SUMMARY

Pathogenic, motile, aerobic sporulating bacilli isolated from a contaminated blood culture and from air and soil were identified as "*Bacillus siamensis*" (Siribaed).

A comparative study of this "*Bacillus siamensis*" group with type cultures of aerobic spore-formers such as *Bacillus cereus*, *Bacillus subtilis*, *Bacillus megatherium* and others showed "*Bacillus siamensis*" to be culturally indistinguishable from *Bacillus cereus*.

Although the "*Bacillus siamensis*" cultures were pathogenic when first isolated, loss of pathogenicity occurred until their virulence was comparable to that exhibited by *Bacillus cereus* after rapid subculture of this species on blood agar.

It is, therefore, concluded that "*Bacillus siamensis*" is identical with *Bacillus cereus*. Since *Bacillus cereus* is one of the most common of the aerobic spore-formers in soil, it is probable that many of the "motile, anthrax-like" and "pathogenic subtilis" bacilli isolated by earlier workers were likewise *Bacillus cereus*.

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ACTIONS TAKEN BY THE SECOND INTERNATIONAL MICROBIOLOGICAL CONGRESS IN LONDON, 1936, REGARDING BACTERIOLOGICAL NOMENCLATURE

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Permanent Secretaries, International Committee on Bacteriological Nomenclature

Received for publication November 16, 1936

The Congress held during the past summer presented the first opportunity for the members of this Committee to meet for conference. The following members of the Committee were present: Prof. S. Orla Jensen (Denmark), Prof. A. Pettersson (Sweden), Prof. F. Mesnil and Dr. A. R. Prévot (France), Prof. M. Grimes (Irish Free State), Prof. A. J. Kluyver (Holland), Dr. J. C. N. Penido (Brazil), Mr. J. Ramsbottom, Dr. Wm. Scott, Dr. A. C. Thaysen, Mr. P. Bruce White and Dr. R. St. John-Brooks (Great Britain), Prof. R. E. Buchanan, Prof. J. Howard Brown, Prof. E. A. Pribram and Prof. R. S. Breed (U. S. A.) Chairman.

Arrangements for two business sessions of the Committee were made at a tea held on Saturday, July 25, at the British Museum of Natural History. At these, there was an interesting discussion of criteria to be used in the classification of micro-organisms led by Mr. P. Bruce White. The Committee then considered the report on proposals of Prof. R. E. Buchanan and Prof. H. J. Conn on the status of the genus *Bacillus* and its type species *Bacillus subtilis* previously published in the Cent. f. Bakt., II Abt., **92**, 1935, 481. The conclusions presented in this report were approved with minor changes for presentation to the plenary session of the Congress.

Dr. St. John-Brooks then presented the report of the *Salmonella* Sub-Committee which had been published in the Journal of Hygiene, **34**, 1934, 333. This embodies the views of the Sub-Committee on the classification of this group, but had not been previously presented to the Committee for action. After discussion, and at the request of the Sub-Committee who desired

the opportunity to carry out further studies, the report was referred back to them for further consideration, with power to fill vacancies on the Sub-Committee, and to co-opt additional members. The Committee expressed its approval of the use of the generic term *Salmonella* with the type species *Salmonella cholerae-suis.*

Prof. Mesnil presented a proposal that would forbid the duplication of generic names among *Protista*, e.g., *Babesia;* and Prof. Breed presented a proposal relative to the non-capitalization of specific names. The actions taken are given below.

Prof. Mesnil was requested to prepare, for presentation at the next Congress, a list of the generic names whose status would be affected by this proposal to invalidate generic homonyms among *Protista.*

Prof. J. Howard Brown was appointed to draw up a statement for presentation to the Committee regarding the use of abbreviations for generic names and was given power to act in appointing a Sub-Committee to work with him.

Dr. Penido suggested that action be taken to establish a more satisfactory nomenclature for viruses. While the group looked with favor on the suggestion, no action was taken as the Committee does not have authority under the rules formulated by the Paris Congress.

The following resolutions were approved at the plenary session of the Congress:

1. On the Proposals of Prof. R. E. Buchanan and Prof. H. J. Conn relative to the status of the Genus *Bacillus* and its type species, *Bacillus subtilis.*
   (a) It was agreed that *Bacillus* Cohn 1873 should be designated as a *genus conservandum.*
   (b) It was agreed that the type species of *Bacillus* should be designated as *Bacillus subtilis* Cohn *emendavit* Prazmowski, 1880.[1]

[1] For subsequent confirmatory and detailed description of *Bacillus subtilis*, see Brefeld, O., Bot. Untersuchungen u. Schimmelpilze, 4, 36, 1881; Gottheil, O., Centralbl. f. Bakt., II Abt., 7, 570, 582, 663, 1901; Conn, H. J., Jour. Infect. Dis., 46, 341, 1930; Conn, H. J., in Bergey's Manual of Determinative Bacteriology, 4 ed., 1934, Baltimore.

(c) It was agreed that the type (or standard) strain should be the Marburg strain.

(d) It was agreed that cultures of the type (or standard) strain of *Bacillus subtilis* together with complete description should be maintained at each of the recognized Type Culture Collections.

(e) It was agreed that the genus *Bacillus* should be so defined as to exclude bacterial species which do not produce endospores.

(f) It was agreed that the term *Bacillus* should be used as a generic name and that it should be differentiated from the terms "bacillus," "bacille" and "Bazillus" used as morphological designations.

2. On the Proposal by Prof. Mesnil relative to generic homonyms.

(a) Generic homonyms are not permitted in the group *Protista*.

(b) It is advisable to avoid homonyms amongst *Protista* on the one hand, a plant or animal on the other.

3. On the Proposal by Prof. Breed relative to non-capitalization of specific names, it was agreed that bacteriologists should accept Article 13 of the International Rules of Zoological Nomenclature, as follows: "While specific substantive names derived from names of persons may be written with a capital initial letter, all other specific names are to be written with a small initial letter. Examples taken from bacteriological literature: *Salmonella Schottmuelleri* or *Salmonella schottmuelleri*, *Bacillus Welchii* or *Bacillus welchii*, *Acetobacter Pasteurianum* or *Acetobacter pasteurianum*, *Corynebacterium ovis*, *Nitrosomonas javanensis*, *Rhizobium japonicum*."

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

CENTRAL NEW YORK STATE BRANCH

THIRTY-SECOND MEETING, CORNELL UNIVERSITY, ITHACA, NEW YORK, NOVEMBER 21, 1936

INFLUENCE OF TEMPERATURE AND HUMIDITY ON THE VIABILITY OF THE OOCYSTS OF EIMERIA TENELLA. *C. C. Ellis*, Veterinary College, Cornell University, Ithaca, New York.

CONFIRMATION OF TESTS FOR THE ESCHERICHIA-AEROBACTER GROUP IN PASTEURIZED MILK. *M. W. Yale*, New York State Agricultural Experiment Station, Geneva, New York. Single tubes of brilliant-green bile (2 per cent) and formate ricinoleate broths were inoculated with 1 ml. quantities of milk from 105 street samples of bottled pasteurized milk representing about 100 different dealers. Both tubes were negative for gas in 76 instances and positive in 23 instances, while in 6 instances gas was produced within 48 hours in one medium but not in the other. Whether the discrepancies were due to false-test organisms or to sampling error was studied by inoculation from the positive tube into a fresh tube of medium which yielded the negative test.

Certain lactose-fermenting spore formers produce false positives in brilliant-green bile broth but not in formate ricinoleate broth when 1 ml. or larger quantities of milk are used. Some members of the genus *Salmonella* produce false positives in formate ricinoleate broth but not in brilliant—green bile broth. Therefore, a negative test obtained from either brilliant—green bile or formate ricinoleate broth following transfer from a positive tube of the opposite medium may indicate the presence of false-test organisms.

When the above procedure was employed in the 6 instances mentioned, positive results were obtained indicating that the original difference in results was due to sampling and not to false-test organisms. Confirmation for members of the *Escherichia-Aerobacter* group showed that they were present in each instance.

False tests occur so rarely where brilliant-green bile or formate ricinoleate broth is employed in the examination of 1 ml. quantities of pasteurized milk that it is not necessary to use more than the presumptive test in practical routine work.

FREQUENCY AND DISTRIBUTION OF THE A FACTOR IN CATTLE. *Alexander Zeissig and C. P. Katsampes*, Veterinary College, Cornell University, Ithaca, New York.

DISCUSSION OF PROBLEMS CONCERNING THE THIRD INTERNATIONAL CONGRESS FOR MICROBIOLOGY. *George P. Berry*, School of Medicine and Dentistry, University of Rochester, Rochester, New York.

DETERMINATION OF ULTRA-VIOLET LIGHT ABSORPTION BY CERTAIN BACTERIOPHAGES. *Leslie A. Sandholzer, Marvin M. Mann, and George P. Berry,* School of Medicine and Dentistry, University of Rochester, Rochester, New York.

MICRO-ORGANISMS ASSOCIATED WITH THE RIPENING OF LIMBURGER CHEESE. *C. D. Kelly,* New York State Agricultural Experiment Station, Geneva, New York.

THE FERMENTATION OF GLUCOSE BY CERTAIN GRAM-POSITIVE BACTEROIDES. *Carl S. Pederson,* New York State Agricultural Experiment Station, Geneva, New York.

THE IMMUNIZING VALUE OF BRUCELLA ABORTUS, STRAIN NO. 19, FOR GUINEA PIGS. *W. S. Stone,* Veterinary College, Cornell University, Ithaca, New York.

STUDIES CONCERNING THE FLAGELLATION OF THE ROOT-NODULE ORGANISM *J. K. Wilson,* Department of Agronomy, Cornell University, Ithaca, New York.

THE TICK AS A VECTOR FOR THE VIRUS DISEASE, EQUINE ENCEPHALOMYELITIS. *J. T. Syverton and George P. Berry,* School of Medicine and Dentistry, University of Rochester, Rochester, New York

ACID-FAST BACTERIA FROM HUMAN SKIN. *Charles M. Carpenter, Margaret M. Andrews, and Lois E. Lange,* School of Medicine and Dentistry, University of Rochester, Rochester, New York.

CHROMOGENIC STRAINS OF ESCHERICHIA CULTURES. *Ralph P. Tittsler,* School of Medicine and Dentistry, University of Rochester, Rochester, New York.

A comparative study reveals that, except for the production of pigment, certain cultures of the *Escherichia* genus do not differ in cultural characteristics from common non-chromogenic types. Four chromogenic strains, three from human feces and one from water, have been tested repeatedly in a wide variety of media during periods ranging from four months to four years. All characteristics remained constant.

All strains produce a reddish-orange pigment at 37°C. and at room temperature. They yield relatively non-pigmented sub-strains which revert to the chromogenic type. All strains produce acid and gas from glucose, lactose, maltose, xylose, arabinose, levulose, mannose, galactose, trehalose, mannitol, sorbitol, and glycerol; produce indol; yield positive methyl-red reactions; and develop typical coli colonies on eosin-methylene-blue agar. None of the strains attack sucrose, dulcitol, erythritol, inositol, cellobiose, α-methylglucoside, inulin, and starch; liquefy gelatin; produce hydrogen sulphide or acetyl-methyl-carbinol; or utilize citrate. On the basis of differences in ability to attack salicin and adonitol, these four cultures divide themselves into three types.

Obviously, these cultures cannot be distinguished from members of the *Escherichia* genus, except on the basis of pigment production. Their similarity to established non-chromogenic species renders the creation of three new species names unnecessary.

THE RELATION BETWEEN THE GROWTH OF MYCOBACTERIUM TUBERCULOSIS AND THE YIELD OF TUBERCULIN ON SYNTHETIC MEDIA

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Received for publication November 17, 1936

In the various synthetic media proposed for growing *Mycobacterium tuberculosis* for tuberculin production, the emphasis has been on finding one which "would yield the greatest amount of growth per unit volume of culture medium in the shortest possible time" (Henley, 1929), on the tacit assumption that the amount of tuberculin produced parallels more or less directly such growth. Thus, Henley and LeDuc (1930), using the weight of organisms produced as a basis of comparison, stated that their ammonium malate medium was somewhat inferior to Henley's asparagine medium. Recently the Committee on Medical Research of the National Tuberculosis Association (White, 1934) adopted a synthetic medium with a high asparagine content for the manufacture of the Purified Protein Derivative (PPD) because it gave "a greater yield in bacilli and production of the desired protein molecule." Substitutes for glycerol in media for tuberculin production have been discarded as unsatisfactory because they yielded fewer organisms. When we reported a synthetic medium from this laboratory (Wong and Weinzirl, 1936) as having the virtue of being inexpensive, we used the weight of organisms produced as the standard of comparison with other media.

Recently, however, because of the increased use of tuberculoprotein antigens in immunizing and desensitizing experiments in the Alice McDermott Foundation, we have been led to raise these questions: What is the relation between the weight of

[1] Now with Peiping Union Medical College, Peiping, China.

organisms produced and the yield of tuberculin on the various synthetic media? Are there other factors than the number of organisms which might influence the yield? A careful survey of the literature reveals very little information on these problems. Seibert (1928), in reporting her method for standardizing Synthetic Medium Tuberculin (SMT), showed a relation between the age of the culture and the amount of tuberculoprotein produced; and in her work on the isolation of PPD (1934) observed that certain strains gave three times as much tuberculin as other strains (within the same type), under conditions so standardized that the same strain in different trials gave practically no variation. However, such contributions have been incidental to other purposes. With the demand for tuberculin as a diagnostic agent, it would appear that the definition of an ideal culture medium, as far as most laboratories are concerned, would need to be altered to "one which would yield the greatest amount of tuberculin per unit volume of culture medium in the shortest possible time," and, incidentally, at the lowest cost.

On this shift in emphasis depend the objects of these experiments: (1) to find an inexpensive synthetic medium for the rapid production of tuberculin; (2) to determine the factors which influence the yield of tuberculin.

METHODS

Organisms used. *Mycobacterium tuberculosis* H 37, grown for a period of two weeks on modified Long's synthetic medium (Wong and Weinzirl, 1936), was used for inoculation throughout the study. When the culture was tested recently, it showed no diminution in virulence for guinea pigs.

Procedures used. (1) The organisms were filtered through a filter paper of the Alpha type in a Buchner funnel, thoroughly washed with distilled water, transferred to an accurately weighed beaker, and dried to constant weight in an oven at 100°C. (2) The amount of protein present in the culture fluid was measured by first centrifuging at 3600 revolutions per minute for half an hour; carefully pipetting the upper layer into a sterile tube; pipetting the desired volume into a Hopkin's vaccine tube; adding an equal portion of 20-per-cent trichloracetic acid; allowing to

stand at ice box temperature overnight; centrifuging rapidly for half an hour before reading the precipitate. (3) The hydrogen-ion concentration was determined colorimetrically.

Preparation of media. Four media were used: (*a*) Long's synthetic medium (Long and Seibert, 1926), (*b*) modified Long's medium (Wong and Weinzirl, 1936), (*c*) modified Henley and LeDuc's medium, prepared according to the directions given by the respective authors, and (*d*) a glucose-sucrose medium.

Modified Henley and LeDuc's medium

Malic acid, the inactive form	10	grams
Ammonium hydroxide (10 per cent solution)	40	cc.
Potassium acid phosphate	3	grams
Sodium chloride	2	grams
Magnesium sulphate	1	gram
Sodium citrate	0.75	gram
Ferric citrate	0.30	gram
Glycerol	50	cc.
Distilled water to make	1000	cc.

After sterilization, 10 cc. of 10 per cent glucose solution, autoclaved separately, is added.

The method of preparation of this medium is essentially the same as of the modified Long's medium previously reported.

Glucose-sucrose medium

Malic acid, the inactive form	10	grams
Ammonium hydroxide (10 per cent solution)	40	cc.
Potassium acid phosphate	3	grams
Sodium chloride	2	grams
Magnesium sulphate	1	gram
Ferric ammonium citrate	0.04	gram
Distilled water	900	cc.

The ingredients may be dissolved by heating. The pH is adjusted to 7.2 and the slight precipitate of ferric phosphate and magnesium ammonium phosphate filtered out. The solution is dispensed in 180 cc. amounts in 500 cc. Florence flasks. After autoclaving at 122°C. for 15 minutes, the pH is about 7.0.

Solution to be added after sterilization:

Glucose	50 grams
Sucrose	10 grams
Distilled water to make	100 cc.

This glucose-sucrose solution is autoclaved in test-tubes containing 20 cc. each, and the contents of a tube added to each flask before inoculation.

RESULTS

Experiment I. The object of this experiment was to compare the weight of the organisms and the amount of protein produced in the various media at the end of 6 and 10 weeks. The results are summarized in tables 1 and 2. The results given in table 1 are the averages of three different flasks selected at random; for the protein determination, duplicates were carried out for each flask, making a total of six determinations. The dried weight of organisms from the glucose-sucrose medium did not vary more than 0.05 gram per flask in the three flasks used. In table 2 the weight of protein given for the glucose-sucrose medium was the average of sixteen determinations on eight different flasks, and that of the modified Henley and LeDuc's the average of six determinations on three different flasks.

TABLE 1

Yield of bacteria and protein at end of six weeks on various synthetic media

MEDIUM	pH	WEIGHT OF DRIED BACILLI CALCULATED PER LITER ORIGINAL MEDIUM	WEIGHT OF PROTEIN CALCULATED PER LITER TUBERCULIN
		grams	*grams*
Glucose-sucrose	7.8	7.15	0.566
Long's*	5.8	13.00	0.189
Long's (modified)*	5 8	10.00	0.222
Henley and LeDuc's (modified)*	6.6	16.50	0.426

* Media containing glycerol.

TABLE 2

Weight of protein obtained at end of ten weeks on various synthetic media

MEDIUM	PROTEIN CALCULATED PER LITER OF TUBERCULIN
	grams
Glucose-sucrose	0.640
Henley and LeDuc's (modified)	0.640
Long's*	0.512

* Seibert's figure (1928) at 12 weeks.

Experiment II. The object of this experiment was to repeat the above experiment employing a larger number of samples and using a different method of approach. The tuberculin and organisms from ten flasks, representing about 2 liters of original medium for each type, were pooled after 6 weeks of growth. The

TABLE 3

Yield of bacteria and protein at end of six weeks

MEDIUM	pH	TOTAL VOLUME	VOLUME LOSS	WEIGHT OF DRIED ORGANISMS	PROTEIN CALCULATED PER LITER ORIGINAL MEDIUM
		cc.	*cc.*	*grams*	*grams*
Glucose-sucrose	7.8	1,545	355	12.62	0.314
Long's	5.8	1,490	410	25.36	0.135
Long's (modified)	5.8	1,525	375	19.40	0.179
Henley and LeDuc's (modified)	6.6	1,420	480	32.16	0.202

TABLE 4

Yield of bacteria and protein at end of ten weeks

MEDIUM	pH	TOTAL VOLUME	VOLUME LOSS	WEIGHT OF DRIED ORGANISMS	PROTEIN CALCULATED PER LITER ORIGINAL MEDIUM
		cc.	*cc.*	*grams*	*grams*
Glucose-sucrose	7.7	1,535	365	10.59	0.449
Long's	5.7	1,450	450	18.87	0.236
Long's (modified)*	5.5	1,475	425	15.32	0.181
Henley and LeDuc's (modified)*	6.8	1,480	420	23.67	0.292

* Filtered through two Mandler filters.

total organisms were well washed with distilled water, transferred to an accurately weighed 400 cc. beaker and dried to constant weight in a 100°C. oven. The pooled tuberculin was treated as follows: the pH and the total volume of the culture fluid were recorded. Then the pH of the other media was adjusted to that of the glucose-sucrose medium with 10 per cent sterile Na_2CO_3 solution and made up to the original volume of the medium before inoculation with sterile distilled water, filtered through Mandler filters, of medium porosity, remeasured and

readjusted to volume with distilled water. The amount of protein was determined in the usual manner. The results given for the tuberculoprotein are the averages of four determinations on the same medium. The culture fluids from the modified Long's medium and the modified Henley and LeDuc's medium filtered so slowly that two candles were employed. The first candles were heavily coated with denatured protein, so the figures given in the last column of table 4 do not represent the true values of the amounts of tuberculoprotein present. A glance at the data presented in table 2 will emphasize this point. No such difficulty was encountered with either the glucose-sucrose or the Long's synthetic medium.

Analysis of the tables presented in experiments I and II reveals the following facts: The glucose-sucrose medium yielded the most protein at the end of six weeks. Its yield at six weeks was greater than that of Long's synthetic medium at the end of twelve weeks. The weight of organisms was approximately half that on Long's medium at the end of six weeks. The modified Henley and LeDuc's ammonium malate medium was superior to Long's synthetic medium in yield of tuberculin and organisms. A larger amount of growth per unit volume of a given medium does not necessarily indicate a greater yield of tuberculin from it.

Experiment III. It does not seem reasonable to find that the yield of synthetic medium tuberculin is independent of the bulk of organisms present when we consider the fact that tuberculin is a composite mixture of the metabolic products of the tubercle bacilli and the products resulting from the autolyzed cells. The discrepancy apparently lies in the rate of cell autolysis, possibly influenced by pH since this differed widely in the various media. Experiment III was designed to test this hypothesis. Modified Long's synthetic medium was selected because of the rapid rate of growth of H 37 on this medium. At the end of four weeks of growth, one set of flasks was adjusted to pH 7.2 with sterile 10 per cent Na_2CO_3 solution and an equivalent volume of sterile distilled water was added to the control flasks. After thorough mixing to insure sinking of the pellicles so that no further growth

of organisms would occur to complicate the results, the flasks were incubated for two more weeks at 37°C. No observable growth took place in that time. The protein determinations were carried out in the usual manner. The results given in table 5 are the average values of duplicate determinations on each flask, a total of six figures for each set of three. The results indicate clearly that an alkaline pH increases the yield of tuberculin. This is in harmony, in principle at least, with the experiment of Hanan and Zurett (1936). In their study of the extraction of protein antigens from *Mycobacterium tuberculosis*, H 37, by means of buffer solutions, these authors showed that a slightly alkaline pH favors the extraction.

Incidental observations on the properties of the glucose-sucrose medium should be included here. Long and Finner (1927)

TABLE 5

The effect of pH on the yield of tuberculin

TOTAL NUMBER OF FLASKS	pH			PROTEIN CALCULATED PER LITER OF TUBERCULIN
	4th week	Adjusted to	6th week	
				grams
3 (control)	5.8	5.8	5.7	0.222
3 (adjusted)	5.8	7.2	8.0	0.471

reported that Long's synthetic medium with 5 per cent glucose replacing glycerol gave only a fifth the bulk of organisms obtained with glycerol. Loebel, Shorr, and Richardson (1933) concluded that glucose could substitute for glycerol to a certain extent, again using the weight of organisms obtained as a basis of comparison.

Maximum growth of *Mycobacterium tuberculosis*, H 37, was obtained in about 6 weeks on the glucose-sucrose medium presented here. The optimum concentration of glucose ranges from 4 to 8 per cent. Concentrations below 4 per cent yielded less than a gram of dried bacilli per 200 cc. of original medium; 4 to 8 per cent inclusive yielded on the average 1.40 grams in 6 weeks; 10 per cent or above inhibited growth. Sucrose alone, whether autoclaved with the medium or added afterwards, did

not support growth in concentrations varying from 1 to 10 per cent. This result is in harmony with the findings of Merrill (1931). The work has been repeated six times with Baker's c.p. sucrose. In no instance was growth observed during 12 weeks of incubation. The presence of the sucrose in the medium appears to increase the yield of tuberculin for a given time. Its precise function is being studied and will be reported in another communication.

The cost of the ingredients in the glucose-sucrose medium as calculated per liter is about 13 cents while that of Long's asparagine medium is about 36 cents. The tuberculoprotein obtained from the glycerol-free medium is apparently identical with those obtained from glycerol media; any suspicion of alteration in specificity due to denaturation at such a high pH has been ruled out by comparative systemic and skin tests made with TPT on sensitive guinea pigs which showed no difference in reaction. Further immunological studies are being made to demonstrate irrefutably the specificity of the protein.

The following species and varieties have been grown: *Mycobacterium tuberculosis*, H 37; *Mycobacterium tuberculosis*, H 1721, from the Michigan Department of Health; *Mycobacterium tuberculosis-bovis*, T 1658, from the University of California; and the non-pathogens from the Phipps Institute, *Mycobacterium phlei*, and *Mycobacterium smegmatis*. When cultures are available other *Mycobacterium* species will be studied.

DISCUSSION

It cannot be denied that the bulk of organisms, the time of incubation, and the rate of evaporation are factors which determine the final yield of tuberculin in any given synthetic medium for growing *Mycobacterium tuberculosis*. Another important factor in the production of tuberculin, at least in the case of *Mycobacterium tuberculosis*, H 37, may be added, a terminal alkaline pH. This conclusion is based upon the following observations: (1) The yield of tuberculin in the modified Long's synthetic medium may be increased twofold merely by adjusting the pH of 5.8 to a pH of 7.2 with sterile 10 per cent sodium

carbonate solution at the end of four weeks of growth and incubating for two more weeks. (2) A glycerol-free medium with a terminal pH of 7.6 at the end of six weeks growth contains about half the number of organisms that Long's synthetic medium will produce, and yet yields approximately three times as much tuberculin as the latter medium.

It was indicated in a previous paper by Wong and Weinzirl (1936) that a glycerol-free medium was in preparation. We felt at that time that the nature of the tuberculoprotein produced in it had not been studied sufficiently to warrant its publication. In the meantime a description of a glycerol-free synthetic medium quite similar to the one presented in this paper has been published by Henley (1935). We have used the latter's medium sufficiently in our laboratory to warrant the restatement of our former objection: the caramelization of the glucose as a result of the autoclaving added an uncertain factor in the changed glucose, which decreased the rate of growth of H 37. No mention was made by Henley of the yield and of the nature of the tuberculin obtained in his medium.

SUMMARY AND CONCLUSIONS

1. There is presented an inexpensive glycerol-free synthetic medium which is suitable for producing tuberculin in large quantities. Approximately three times as much protein may be obtained from the glucose-sucrose medium as from Long's synthetic medium at the end of six weeks. The cost of production of the medium is about a third of that of Long's medium.

2. *Mycobacterium tuberculosis*, H 37, produces a progressive alkalinity on the glucose-sucrose synthetic medium.

3. A terminal akaline pH is an important factor in the rapid production of tuberculin in the case of *Mycobacterium tuberculosis*, H 37.

4. Glucose is a satisfactory substitute for glycerol in growing *Mycobacterium tuberculosis*, H 37, under the conditions specified in the report. It is better than glycerol in the production of synthetic medium tuberculin.

The author gratefully acknowledges the technical assistance of Miss Doris J. Hirschmann in running the pH determinations.

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RELATIONS BETWEEN PLATE COUNTS AND DIRECT MICROSCOPIC COUNTS OF ESCHERICHIA COLI DURING THE LOGARITHMIC GROWTH PERIOD[1]

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Received for publication August 8, 1936

INTRODUCTION

The relation between the plate count and the direct microscopic count of bacteria is of practical as well as theoretical importance. Particularly during the logarithmic growth period, where the constant rates are used for comparative purposes, it is desirable to know whether the number of cells as determined by plating always bear a constant relation to those enumerated microscopically, that is, whether the plate count is a reliable, although relative, measure of the total number of cells present.

That the plate count is usually lower than the total count was noted years ago (Winterberg, 1898; Klein, 1900; Hehewerth, 1901), and this discrepancy has been the subject of a relatively few papers since that time. In general, are the differences between such counts, even during the logarithmic growth period with pure cultures, due entirely to clumping, or to the death of a certain proportion of bacteria, or to a combination of these factors? The more recent studies have brought various facts to light in respect to some of the relationships and have offered explanations in certain cases, without having clearly answered these questions. It is recognized, for example, that in old cultures many cells are not viable, but it is uncertain whether or not this is the case during the period of logarithmic reproduction in a suitable medium.

[1] Contribution No. 89 from the Department of Biology and Public Health, Massachusetts Institute of Technology.

Some confusion exists in the literature in regard to the use of the term "total count." Some authors use it literally as meaning that each and every individual cell is counted as one, which is the terminology employed in this paper, while others use it to include both single cells and clumps of bacteria which theoretically give rise to one colony upon plating. In some cases the terms "direct count" and "microscopic count" have been used without further specification. Moreover, there have been apparently unsupported statements that in rapidly-reproducing cultures there is no clumping, and data have sometimes been compared on this assumption. That there is actually a good deal of clumping in young cultures, even with the reputedly "individualistic" organism *Escherichia coli*, has been shown definitely by Glynn, Powell, Rees and Cox (1913), Knaysi (1935), Ziegler and Halvorson (1935), and by our own observations. The clumps, which are not easily broken up by shaking, are most commonly of two organisms, and probably result from incomplete separation after fission. These clumps, alone, if not considered, are sufficient to cause marked discrepancies between plate and total count data. Unless all clumps can be completely broken up, the only fair basis for comparison of the relation between the plate and direct counts—at least so far as viability is concerned—is to use for the direct enumeration a microscopic "group count," in which each clump of two or more cells, as well as single organisms, is counted as one unit.

The explanations of the discrepancies noted by various investigators between plate and microscopic counts may be considered to fall into three classes: (1) counting errors (due to chance) involved in the two methods; (2) clumping; (3) variations in resistance of cells, in which case some cells die either in culture, during the process of plating, or on an unsatisfactory plating medium. A uniform technique, to minimize errors other than those due to chance, is assumed. As regards counting errors, the reliability of results in a given case can be determined statistically, although such data have usually been lacking in published reports. Under the proper conditions the accuracy of bacterial counts obtained either from plates or by direct microscopic enu-

meration, particularly with a Helber type counting chamber, is about the same (Wilson, 1922; Wilson and Kullmann, 1931), and this may be assumed here for purposes of discussion. In respect to clumping, it must be assumed that the number of clumps when the bacterial suspension is examined microscopically is the same as when plated, taking into account the dilution factor.

Various aspects of the effect on the plate count of the diluent used in plating have been discussed by Wilson (1922), Cohen (1922), Falk (1923), Winslow and Falk (1918, 1923), Shaughnessy and Criswell (1925), Winslow and Brooke (1927), and Butterfield (1932). It is clear that there are marked differences in viability between various species exposed to different diluents, often during a period of only a few minutes.

Brew (1914), using the microscopic method of Breed (1911), with milk of various ages, found the microscopic (individual) cell count to be ten or more times greater than the plate count, due largely to clumping. Microscopic group counts were about twice the plate counts, which he considers as probably due to the fact that the agar was not optimum for the development of all types of milk bacteria. Conn (1915), and Baker, Brew and Conn (1920), using the Breed method, showed a reasonably good agreement between the group count and the plate count in milk.

Glynn, Powell, Rees and Cox (1913), with a Helber cell for direct counts, found the group count of staphylococci and *E. coli* somewhat larger than the plate count, the difference becoming progressively greater with increasing age of culture, presumably as a result of more organisms becoming moribund. During the logarithmic growth phase, at optimum temperature, the plate count was from 5 to 30 per cent less than the group count.

Reichenbach (1911), in order to explain the logarithmic order of death, assumed that a certain proportion of the multiplying bacteria of each new generation ceased reproduction and became dormant. Wilson (1922, 1926) determined with pure cultures of *Salmonella suipestifer* and *Salmonella aertrycke*, that the viable count averaged 80 per cent of the total count (with a Helber cell) during the logarithmic period at optimum temperature, although in a few instances results were identical. His explana-

tion of the discrepancy also is that some young cells are actually dying out *in the original culture.*

That some bacteria may be killed during plating, may be inferred from the work of Sherman and Albus (1923, 1924), in which young cultures showed less resistance to temperature and osmotic pressure; young cells are also more easily killed by abrupt environmental changes (Sherman and Cameron, 1934). On the other hand, Barber (1908) found that, with proper care in transferring, single cells of *E. coli* from young cultures were nearly 100 per cent viable. The work of Chesney (1916) on the growth of pneumococcus tends to show that these cells do not die during the logarithmic phase either in culture or upon transplanting, if a favorable environment is present.

Jensen (1928) determined the plate count to be lower than the total count, even during the logarithmic phase. Using Ørskov's (1922) method of direct observation of cells on agar blocks, he followed the growth of cells of *E. coli* from an 18-hour broth culture, and found the original cells and their progeny to have 100 per cent germination until about the end of the logarithmic growth period, after which time there was a decreased reproductivity due to the production of "shadow forms." If cells were transferred during the phase of maximum rate of reproduction to a new medium, some were killed, indicating susceptibility to changes in environment. Kelly and Rahn (1932) by direct observation on agar blocks of single cells of *Aerobacter aerogenes*, *Bacillus cereus* and *Saccharomyces ellipsoideus* from young cultures, found practically 100 per cent germination, infant mortality being nil.

Anderson, Fred and Peterson (1920), in comparing the direct group count with plate counts of *Lactobacillus pentoaceticus*, found the two essentially the same during the logarithmic phase. With increasing age, the plate count became progressively lower than the group count. This progressively greater difference with increased age of culture was also shown for *E. coli* by Henrici (1928), and for *Pseudomonas aeruginosa* by Régnier, David and Kaplan (1932). Ziegler and Halvorson (1935), using for the direct

count the smear method of Henrici (1923, 1928), compared the plate count of *E. coli* with the total count after shaking thoroughly to break up clumps, and concluded that the two are practically identical before the death phase.

The reliability of staining methods used to differentiate between dead and living bacteria has been seriously questioned by Bickert (1930) and others. The data on viability, obtained by tinctorial reactions, are contradictory, but for the sake of completeness, a few recent papers may be mentioned. Gay and Clark (1934), using the Proca-Kayser technique to stain living and dead bacteria, differentially, studied the viability of several forms, and found that even in cultures five hours old there were frequently many cells which showed the color typical of dead organisms. On the other hand, Henrici (1928, Ch. 9), found that cells of the colon bacillus did not stain with Congo red until more than twenty-four hours old. Knaysi (1935) criticizes the Proca-Kayser technique, and recommends neutral red for the same purpose. Comparing the group count of viable cells, as shown by his technique, with the plate count of *E. coli*, he finds that during the logarithmic growth phase there is very close agreement between the two; furthermore, as shown by staining, there are not more than one or two per cent of dead cells present during this period, the percentage increasing with age (personal communication).

PURPOSE

The present study was undertaken to determine the relations between the plate count, the group count, and the total count of *Escherichia coli* during the period of maximum rate of reproduction (logarithmic growth phase), under the best practical conditions. A statistical analysis of the data is included, to indicate the reliability of the results. The organism used was chosen partly because the cells show less tendency than in many species to remain attached together after fission, and partly because its resistance to slight changes in environment and its good growth on artificial media were desirable characteristics. For the purpose of determining whether the temperature of growth has any

influence on viability in culture, several incubation temperatures were employed. The effect on the plate count of the dilution fluid was first investigated.

EFFECT OF THE DILUENT ON THE PLATE COUNT

Preliminary experiments were made on the viability in various dilution fluids of the strain of *E. coli* used, in order to rule out as far as possible the death of cells during plating. Distilled water (pH 6.4) at 22°C. was found to be reasonably satisfactory for this organism, as follows.

Shaken (1 minute), pooled, duplicate broth cultures of known age were serially diluted to a predetermined number of cells (200 to 300 per ml.) convenient for plating and counting. The dilution

TABLE 1

Effect of time of exposure on survival of Escherichia coli in distilled water at 22°C., as determined by plate count

AGE OF CULTURE	TIME OF EXPOSURE, MINUTES					
	0 (5)	10	15	20	25	30
	Per cent survival					
hours						
3	100	99	98	97	97	98
6	100	95	96	94	94	89

was made and plated within five minutes, and the mean count of triplicate plates taken as the initial (0(5) minute) reading. This same dilution was allowed to stand at 22°C., and plated (within three minutes) after various periods of time up to thirty minutes, the same pipette being used at each time interval. Pyrex test tubes and pipettes were employed throughout. Tubes of broth, dilution water, and pipettes had an error of ±1.0 per cent by volume. Typical data are given in table 1.

This table shows the survival in distilled water at 22°C., for various periods of time, of three-hour and six-hour cultures of *E. coli*. Here the initial plating is taken as 100 per cent. Calculation of the standard deviation of these mean counts, and using plus or minus twice the standard deviation as the criterion of the prob-

able limits of the means, showed that the apparent decreases were within the experimental error. These decreases therefore are without statistical significance, but the data at least indicate a tendency, since in all of many similar experiments the later counts were invariably lower than the initial.

There is also a possible indication from this and other data not presented, that older (six-hour) cultures are apparently more susceptible to the diluent than younger (three-hour) cultures. It should be noted, however, that the three-hour culture was only diluted 1:1000 for plating, while the six-hour culture required a dilution of 1:100,000. It is generally recognized that young cultures are *more* susceptible to environmental changes than old cultures, and in our case the explanation of this apparent paradox may be that the "colloid protective substance" (Winslow and Brooke, 1927) of the original broth is more highly diluted in the old cultures and thus has less protective action over a period of time.

These experiments indicate that even under practical conditions the time of exposure to the diluent may be an important factor in plating, and should be kept low.

RELATIONS BETWEEN PLATE AND DIRECT COUNTS

Methods

A stock culture of *Escherichia coli* (C_1), which was reproducing at a maximum rate in broth at 22°C., was used throughout the work. Two series of plate and direct count data were obtained, the platings at the four temperatures in each series being done on the same day under the same conditions.

Series of culture tubes containing 9 ml. of nutrient broth were inoculated with the same known number of cells (about 2000 per milliliter) from a twelve-hour, 22°C. broth culture. The tubes were incubated at 22°, 27°, 32° and 37°C. At exactly two-hour intervals, two (new) tubes were removed at each temperature, stoppered with rubber stoppers and shaken by hand (without beads) for one minute, and the contents pooled before sampling. A portion was immediately preserved with formalin for the direct count, and another portion serially diluted in 9 ml. distilled water

blanks (pH 6.4) at 22°C., and plated within five to ten minutes, five plates being made from the proper dilution. Plates were incubated for 48 hours at 37°C. before counting, and the dilution giving 100 to 400 colonies per plate—within which range Wilson (1922) has shown the balance between errors of sampling and of over-crowding to be optimum—was used for enumeration. Most of the mean counts reported are averages of five plates, which number is adequate for precise work (Wilson and Kullmann, 1931).

Conditions of incubation, dilution, etc., were controlled so as to minimize experimental errors. Temperatures did not vary more than ± 0.15°C. from those specified, except that of the dilution water which had a variation of ± 0.5°C. All water blanks and tubes of broth contained 9.0 ml. ± 0.1 ml. of liquid when used. The same lot of Difco dehydrated broth and agar was used throughout, agar being poured at 42°C. The average variation in 1 ml. dilution pipettes was ± 1 per cent by volume.

For the direct counts, a Helber counting cell 0.02 mm. deep was used, the organisms being examined in a light field after staining by the addition of methylene blue to the formalinized suspension. For most sampling periods the organisms in 400 small squares of the chamber were counted; this was varied in a few cases when the organisms were too few or too numerous. The number of cells or groups enumerated in each sample varied, except in a few early counts of about 200, from 500 to 3600, which latter are adequate for a precise mean (Wilson and Kullmann, 1931). Two sets of direct counts were made at the same time on each sample—a *total count*, in which each individual cell, whether in a clump or not, was counted as one, and a *group count*, in which each single isolated cell and each clump of two or more cells was also counted as one. It must be assumed that the number of clumps were relatively the same in these suspensions as in the diluted portions which were plated.

Statistical treatment

With a standardized technique, under conditions giving small and constant experimental errors, the chief errors of the estima-

tion of bacterial populations are those due to chance, and the accuracy depends upon the number of cells or colonies counted (Fisher, Thornton and MacKenzie, 1922; Fisher, 1930; Wilson and Kullmann, 1931).

From the five replicate plate counts at each sampling interval, the standard deviation of the mean (standard error) was calculated by the usual statistical formula. These standard errors (coefficients of variation) varied from $\pm$ 0.5 to $\pm$ 8.3 per cent, and averaged $\pm$ 4.0 per cent of the means for the whole series.

The standard error of direct counts is given by $\pm \sqrt{m}$, where m is the number of cells or groups enumerated (Fisher, 1930, p. 57). The standard errors of the *total* counts were between $\pm$ 1.7 and $\pm$ 7.7 per cent of the counts, with an average value of $\pm$ 3.6 per cent. The *group* count error varied from $\pm$ 1.9 to $\pm$ 8.2 per cent, averaging $\pm$ 4.2 per cent of the counts. These direct count deviations are, on the average, of about the same order of magnitude as those of the plate counts, and the accuracy of counts by either method is therefore about the same.

Relations between different kinds of counts were expressed as percentage ratios. The standard error of each ratio was given by the square root of the sum of the squares of the standard errors of numerator and denominator, and plus or minus twice this standard error taken as the reasonably certain maximum variation of the ratio. The probable limits of the ratios, using this criterion, are shown in tables 2 and 3.

Series I

The bacterial counts, when plotted against time on semi-log paper, gave typical sigmoid growth curves (Jennison, 1935). Because of the fact that during the earliest part of the logarithmic growth period the density of cells was too low to make accurate direct counts, the data for comparison here include the latter half of this phase and one subsequent two-hour growth interval at each temperature in tables 2 and 3.

Table 2 gives the total counts, microscopic group (single cells and clumps) counts, and plate counts for the first series of experiments, together with an analysis in the form of ratios to bring

out the relations involved. The data are comparable, since the same number of cells was used for the original inoculation at each temperature, and the same lot of broth for all cultures.

As is to be expected, the plate count (actually a viable group count) is invariably lower than the total, as shown by the plate/total ratios. That these ratios are statistically significant is seen from their probable limits of variation, the ratios not reaching

TABLE 2

Total counts, group counts, and plate counts of Escherichia coli in broth at various temperatures, during approximate logarithmic growth period (Series I)

TEMPERATURE	AGE OF CULTURE	BACTERIA PER MILLILITER, THOUSANDS			RATIOS AND THEIR REASONABLY CERTAIN (TWICE STANDARD ERROR) LIMITS		
		Total count	Group count	Plate count	$\frac{\text{Plate}}{\text{Total}} \times 100$	$\frac{\text{Group}}{\text{Total}} \times 100$	$\frac{\text{Plate}}{\text{Group}} \times 100$
°C.	hours						
37°	4	3,880	2,980	2,350	61 (52–70)	77 (61–93)	79 (66–92)
	6	101,000	74,200	60,000	59 (57–61)	73 (69–77)	81 (76–86)
	8	166,000	127,000	126,000	76 (68–84)	77 (72–82)	99 (89–109)
32°	6	10,000	6,620	5,870	59 (50–68)	66 (53–79)	89 (78–100)
	8	88,300	72,000	72,000	82 (77–87)	82 (79–85)	100 (93–107)
	10	121,000	109,000	95,000	79 (66–92)	90 (85–95)	88 (74–102)
27°	8	5,220	4,630	3,500	67 (53–81)	89 (70–108)	76 (59–93)
	10	32,000	27,200	24,400	76 (68–84)	85 (75–95)	90 (80–100)
	12	107,000	95,600	90,000	84 (76–92)	89 (83–95)	94 (84–104)
	14	144,000	130,000	117,000	81 (76–86)	90 (85–95)	90 (84–96)
22°	14	9,000	6,770	6,900	77 (60–94)	75 (60–90)	102 (82–122)
	16	33,600	19,500	20,100	60 (53–67)	58 (50–66)	103 (89–117)
	18	57,700	36,400	43,000	75 (63–87)	63 (57–69)	118 (98–138)
	20	142,000	99,300	100,000	70 (64–76)	70 (66–74)	100 (92–108)
	22	171,000	132,000	129,000	75 (66–84)	77 (72–82)	98 (86–110)

100 per cent in any case. There is no evidence that temperature has any marked effect on the ratios. The plate counts as determined vary from 59 to 84 per cent of the total at various ages and temperatures, giving a mean of 72 per cent (with average probable limits, using the criterion of twice the standard error, of 63 to 81 per cent). Since the viable group count accounts for only 72 per cent of the organisms, the remaining 28 (19 to 37)

per cent of the bacteria must be *either members of clumps* (these clumps being already enumerated in the viable group counts), *or non-viable cells.* Now, the microscopic group count averages 77 (average limits, 68 to 86) per cent of the total, the remaining 23 (14 to 32) per cent of the bacteria being members of clumps enumerated in this count. Since there is no significant difference between the figure of 28 per cent (cells either in clumps or dead) and the value of 23 per cent (cells in clumps), the differences between plate and total counts may be explained by *clumping* rather than by the *death* of some cells.

TABLE 3

Total counts, group counts, and plate counts of Escherichia coli in broth at various temperatures, during approximate logarithmic growth period (Series II)

TEMPERATURE	AGE OF CULTURE	BACTERIA PER MILLILITER, THOUSANDS			RATIOS AND THEIR REASONABLY CERTAIN (TWICE STANDARD ERROR) LIMITS		
		Total count	Group count	Plate count	$\frac{\text{Plate}}{\text{Total}} \times 100$	$\frac{\text{Group}}{\text{Total}} \times 100$	$\frac{\text{Plate}}{\text{Group}} \times 100$
°C.	*hours*						
37°	6	16,200	7,300	7,100	44 (39–49)	45 (37–53)	97 (83–111)
	8	146,000	90,000	90,000	62 (57–67)	62 (57–67)	100 (90–110)
	10	193,000	134,000	134,000	70 (65–75)	70 (65–75)	100 (92–108)
32°	8	15,500	11,300	8,500	55 (48–62)	73 (60–86)	75 (64–86)
	10	145,000	77,000	73,000	50 (47–53)	53 (48–58)	95 (91–99)
	12	171,000	148,000	146,000	85 (78–92)	87 (81–93)	99 (90–108)
27°	10	4,600	2,600	2,200	48 (42–54)	57 (45–69)	85 (71–99)
	12	28,600	15,500	16,000	56 (48–64)	54 (46–62)	103 (86–120)
	14	104,000	73,700	75,000	72 (62–82)	71 (67–75)	102 (88–116)
	16	172,000	116,000	119,000	69 (62–76)	67 (62–72)	103 (97–109)
22°	14	43,500	31,400	10,100	*23 (21–25)	72 (64–80)	*32 (29–35)
	16	59,000	46,000	39,000	66 (55–77)	78 (68–88)	85 (71–99)
	18	141,000	111,000	98,000	70 (70–75)	79 (73–85)	88 (81–95)
	20	220,000	161,000	143,000	65 (60–70)	73 (67–79)	89 (81–97)

* Omitted from averages.

The group/total ratios, which would approximate unity with less clumping, demonstrate also the very considerable number of groups of cells, which were not even broken up by shaking, present during the logarithmic phase. The great majority of these

clumps, as noted microscopically, were due to so-called biological clumping (incomplete separation of cells after fission), as contrasted with a few larger masses of entangled cells.

The plate/group ratios, in themselves, show only that all of the *groups* (single cells and clumps) present in the original culture will grow on plates, but considered in conjunction with the other ratios, indicate viability of *individual* organisms. Considering the possible errors involved, and the fact that the groups determined microscopically may not be exactly the same in number as those plated, the plate and microscopic group counts show very good agreement, not only on the average, but individually as well. At 37°C., the plate count averages 86 (limits, 76 to 96) per cent of the group; at 32°, 92 (81 to 103) per cent; at 27°, 88 (76 to 100) per cent; and at 22°, 106 (91 to 121) per cent of the group. The average indicated viability in the four experiments is about 94 (83 to 105) per cent, which, within the limits of error of the experiments, is equivalent to practically 100 per cent viability for individual organisms.

Series II

The data and ratios for the second series of experiments—all the culturing and plating for which was done on the same day with identical materials—are shown in table 3. In this case, as in the previous series, the plate count is always significantly lower than the total, varying (omitting the 22°, 14-hour ratio, which is so far off as to indicate a probable technical error) from 44 to 85 per cent, and averaging 62 (average limits, 55 to 69) per cent, of the total under various conditions. In the same manner as before, the other 38 (31 to 45) per cent of the organisms must be either members of clumps or non-viable. The group count averages 67 (limits, 60 to 74) per cent of the total, leaving 33 (26 to 40) per cent of the cells as members of these microscopic groups. In this case also, the difference between 38 and 33 per cent is not significant, and the discrepancy between plate and total count can be wholly explained by clumping of cells.

The plate count at 37° in this case averages 99 (limits, 88 to 110) per cent of the group; at 32°, 90 (82 to 98) per cent; at 27°,

98 (84 to 112) per cent; and at 22°, 87 (77 to 97) per cent (omitting the 14-hour ratio) of the group. In series II also, the evidence is reasonably clear that practically all of the bacteria in the culture will grow on plates, the average viability in the four experiments being 92 (82 to 102) per cent.

DISCUSSION

The question as to whether an appreciable proportion of bacteria are dying out *in culture*, at various temperatures, during the logarithmic period seems to be answered conclusively in the negative. The discrepancies between plate counts and total counts can be wholly explained by clumping of organisms, as shown by the group/total ratios which are essentially the same as the plate/total figures, in most individual cases as well as on the average. The plate/group ratios then, can be taken as a measure of the viability of individual cells. With one or two possible exceptions they indicate, within the limits of error of the methods, a viability of 100 per cent for *E. coli* during the logarithmic growth phase.

As regards the few exceptions, all of which are very nearly within the limits of experimental error, we believe them to be more probably due to technique, where some organisms die during plating, rather than to the fact that some cells are dying out *in culture*. The following indicates the basis for this interpretation.

In the first place, plate counts are more likely to be too low rather than high, due to the death of a certain number of cells especially susceptible to environmental changes, as evidenced by the work of Sherman and Albus (1923, 1924), Jensen (1928), Sherman and Cameron (1934), and others previously cited. Most studies, including our own, on survival of bacteria in diluents also indicate a tendency in the direction of a decrease in numbers of cells, if there is any change at all, rather than the reverse. Secondly, the investigations of Barber (1908), Jensen (1928), and Kelly and Rahn (1932), in which single cells from young cultures were transferred to favorable environments and observed, point to practically 100 per cent viability *in culture*. The evidence on viability, obtained from differential staining of dead and living cells, is considered unreliable for accurate work.

Several investigators state that the ability of organisms to take up stains merely shows different degrees of destruction of cells, although loss of reproductive power usually occurs before staining (Fraser, 1920; Henrici, 1928; Rahn and Barnes, 1933; Knaysi, 1935). Certainly staining is a less direct method than plating to determine viability, and it is felt that at present more weight should be given to plating and direct counting in interpreting viability data.

It appears to us that the most direct evidence indicates that during the logarithmic growth phase, in a suitable medium, all of the cells of *E. coli* are viable. There may be a tendency for some to die during plating if conditions are not optimum. With bacteria which are more susceptible to environmental changes, it is quite possible that a certain proportion may die in culture. It is perhaps more likely, however, that such organisms would die during plating, in which case the evidence for mortality in culture would be indirect, being dependent upon the limitations of experimental technique.

With as "individualistic" an organism as the colon bacillus there was a considerable amount of clumping in broth cultures during the logarithmic phase of growth, as indicated by the average group/total ratio (either microscopic group or viable group), after shaking, of about 70 per cent. The clumps—which were mostly of two cells, at least after shaking—were broken up to a varying extent by the shaking. Largely, at least, because of different amounts of clumping, the ratio of plate count to total (individual) cell count varied to the extent of about 100 per cent with different sampling intervals. Since the plate/total ratio was not constant—witn clumps present—throughout the period of logarithmic growth, the plate count under these conditions cannot be considered to be a very reliable measure of the number of organisms relative to the total. Having established that practically all of the individual bacteria are viable, it appears that if clumps could all be broken up the plate counts would be identical with the total counts, within the limits of experimental error. This conclusion was also reached by Ziegler and Halvorson (1935), for *E. coli* cultures before the death phase.

It should be so obvious as not to require mention, that in viability studies the question of clumping must be taken into account in one way or another, although strangely enough many investigators apparently have not considered it of quantitative significance.

CONCLUSIONS

For *Escherichia coli*, in broth culture at different temperatures from 22° to 37°C., the following conclusions may be drawn relative to its behavior during the logarithmic growth period.

1. Even in young cultures there is considerable clumping, the group counts having averaged 70 per cent of the total counts, after shaking.
2. The discrepancies between plate and total counts can be wholly explained, within the limits of experimental error, by clumping of cells, other conditions being optimum.
3. The plate count—due to the varying amounts of clumping at different times—does not bear a constant relation to the total (individual) cell count throughout the logarithmic period. When there is clumping, therefore, a plate count *may* not be a reliable measure of the number of cells present relative to the total.
4. If groups of bacteria can be completely broken up, the plate and total counts will be practically identical during the period of maximum rate of reproduction.
5. Different temperatures of growth seem to have no significant effect upon the viability of these bacteria in culture.
6. The results indicate practically a 100 per cent viability *in culture* for this organism. There may be a tendency for some cells to die during the plating-out process.

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STUDIES ON THE EFFECT OF SYNTHETIC SURFACE-ACTIVE MATERIALS ON BACTERIAL GROWTH. II

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Received for publication October 20, 1936

In a previous communication (Katz and Lipsitz, 1935), the effect of a certain synthetic surface-active material on the growth of *Mycobacterium smegmatis* was discussed; this work has now been extended to include three more compounds, varying in chemical structure. These materials possess substantially the same properties as the alkylated naphthalene sulfonate originally employed, i.e., remarkable capillary-activity plus sufficient chemical stability to retain capillary-active properties in solutions of low pH and in the presence of alkaline earths and heavy metal salts.

MATERIALS

The compounds utilized are colloidal electrolytes; a general discussion of these materials is to be found in the Symposium on Colloidal Electrolytes of the Faraday Society, particularly in the paper of Stewart and Bunbury (1935). They are amorphous solids and are difficult to characterize by criteria usually employed for crystalline solids. While the exact molecular configuration is as yet undetermined for compound A, the methods of preparation employed in the synthesis of all these compounds yield definitely reproducible compounds. Full details of preparation may be obtained from the appended references.

A. Mono-sodium sulfonate of butylated o-hydroxy diphenyl. This product was free from all but traces of sulfate and chloride.[1]

B. This compound is a quaternary ammonium salt, prepared

[1] U. S. 1,994,927.

by the reaction of dimethyl sulfate with the stearyl ester of unsymmetrical diethyl ethylene diamine.[2] The product was free from all traces of inorganic salts, and yielded no ash on ignition. It is of interest in view of recent work on the antiseptic properties of quaternary ammonium salts (Domagk, 1935).

C. The N-methyl tauride of oleic acid.

$$\begin{array}{ccccc} & & CH_3 & & \\ & & | & & \\ C_{17}H_{33}C & — & N & — & C_2H_4SO_3Na \\ \| & & & & \\ O & & & & \end{array}$$ [3]

The product employed consisted of 33.5 per cent active material, 2.5 per cent sodium oleate, 8 per cent sodium sulfate, 56 per cent water (Lederer, 1934). While this is not a pure compound in the sense of being a distinct chemical individual, yet it is a perfectly reproducible mixture. To be precise, we must consider the effect of this compound to be the resultant of the action of its various components; in the dilutions employed, however, the concentrations of the accompanying materials are below those usually recognized to have an effect on bacterial growth.

The first compound is a cyclic derivative very similar to the compound employed originally. The last two compounds are distinguished by the presence of long aliphatic chains in their molecules. Compound B differs from A and C in that capillary activity, in the case of B, is resident in positively charged colloidal micelles; in the case of the other two materials, capillary activity is associated with the presence of negatively charged colloidal micelles.

EXPERIMENTAL

All technique employed was the same as in the original work. Bacterial growth was checked by visual observation and by microscopic examination. Smears were made from the original cultures at 12, 24, and 36 hours. Transfers into plain glycerol broth were made at the end of 12 hours.

[2] D. R. P. 430,090; 464,142; 535,061. U. S. 1,737,458.
[3] Fr. 693,620; Brit. 341,053; 343,524.

Microscopic examination did not reveal any morphological changes; whenever the smears disclosed bacteria, they were apparently normal organisms.

The results so far obtained seem to indicate the greater effectiveness of cyclic compounds as contrasted to aliphatic compounds. The possession of a positively charged colloidal micelle does not seem to impart any particularly outstanding properties, at least insofar as a heavily capsulated organism is concerned.

TABLE 1

	DILUTION OF SURFACE TENSION DEPRESSANT	12 HOURS	24 HOURS	36 HOURS	INOCULATED INTO PLAIN BROTH
A	1:100 to 1:2,000	—*	—	—	—
	1:3,000 to 1:12,000	—†	—	—	+
	Above 1:15,000	+	+	+	+
B	1:100 to 1:1,000	—*	—	—	—
	1:1,000 to 1:2,000	—†	—	—	+
	Above 1:2,000	+	+	+	+
C	1:100 to 1:2,000	—*	—	—	—
	Above 1:2,000	+	+	+	+

* Pellicles sank.
† Growth inhibited.

CONCLUSIONS

1. The effect of three synthetic surface-active materials on the growth of *Mycobacterium smegmatis* has been determined.
2. Results so far obtained indicate that those surface-active properties associated with possession of a cyclic structure are more effective in inhibiting growth than those associated with possession of long aliphatic chains.

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STREPTOCOCCUS ZYMOGENES

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Received for publication November 25, 1936

MacCallum and Hastings (1899) described under the name of *Micrococcus zymogenes* an organism obtained from a case of acute endocarditis. They noted the characteristic grouping in pairs, less frequently in short chains, and stated that the organism showed points of resemblance to *Streptococcus pyogenes* and the pneumococci on the one hand and to the pyogenic staphylococci on the other. MacCallum and Hastings indicated that their organism was probably a common intestinal form, as evidenced by the fact that it had also been isolated from sewage by their associate, Dr. Norman MacLeod Harris. Shortly after the work of MacCallum and Hastings, Harris and Longcope (1901) reported the organism in specimens from human autopsies, and since that time it has been reported intermittently from clinical and fecal sources. This organism was early recognized as a streptococcus (Winslow and Winslow, 1908) and has been so classified by more recent investigators.

Although it cannot be certain that all cultures that have been called *Streptococcus zymogenes* by subsequent investigators have in fact been the organism of MacCallum and Hastings, there would appear to be little room for error in its identification. MacCallum and Hastings' description is an extraordinarily beautiful example of the accuracy with which the characteristics of bacteria were sometimes portrayed by the earlier bacteriologists, whose working tools were so limited in comparison with the methods of today. The liquefaction of gelatin and the proteolysis of casein were noted; attention was called to the strong reducing action of the organism in litmus milk, the especially significant point being

observed that complete reduction of the litmus took place before acidulation and curdling; and, though they worked before the days of the now familiar fementation tests, it was noted that the organism grew more profusely on glycerol agar than on ordinary nutrient agar, plainly indicating that glycerol was utilized. The hemolysis of blood as a test in the study of the streptococci was of course not in use at that time. Within the limits of present knowledge of the streptococci, the only adequately described organisms which fulfill entirely the combination of characteristics given by MacCallum and Hastings are the hemolytic *Streptococcus zymogenes* and the non-hemolytic type which is frequently designated as *Streptococcus liquefaciens*.

We have had a unique opportunity to study *S. zymogenes*, as one of our associates, up to recent months at least, appeared constantly to harbor this organism in appreciable numbers in his intestinal tract. The organism could not be isolated at will by direct plating of the stools, but frequently could be so isolated, and usually with ease, in periods of intestinal disturbance.

There would probably be little point in making a study of a large collection of cultures isolated from a few samples from this one source. As the cultures obtained from any one sample would be apt to represent only different isolations of one strain, such a study might give a false impression of homogeneity in the group represented by the organism. We have subjected to detailed study a small collection of 26 cultures which were isolated from many different samples over a period of nearly ten years. In addition, we have studied certain characteristics, especially the proteolytic and hemolytic properties, of a large number of additional cultures. At the same time, we have studied another collection of the non-hemolytic *S. liquefaciens* obtained from other fecal sources and from milk, but this organism will be brought into the discussion only incidentally.

In the present paper we wish to record a somewhat more detailed description of *Streptococcus zymogenes* than is now available; to bring out more clearly its relationship to *Streptococcus fecalis* and its status as an "enterococcus;" and to present some information dealing with the apparent variation in its proteolytic properties, as revealed by different strains.

STREPTOCOCCUS ZYMOGENES AS AN "ENTEROCOCCUS"

The term "Enterococcus" has been used in a more or less specific sense for *Streptococcus fecalis*, but, in recent years at least, it has more frequently been applied in a rather loose group sense to those fecal streptococci of which *S. fecalis* is the best known and central type. In this connection, the review by Dible (1921) will be found of interest. There appears to be some point to an "enterococcus group" to encompass *S. fecalis*, *S. zymogenes*, *Streptococcus liquefaciens*, and probably other species, as yet not clear, having the same basic characteristics which differentiate them from the other important groups of the streptococci.

Outstanding among the characteristics of *S. fecalis* and its related enterococci are: low minimum and high maximum temperatures of growth, high thermal resistance, strong reducing action, the production of a low final pH value in glucose broth, the production of ammonia from peptone, and the hydrolysis of esculin. *S. zymogenes* has all of these characteristics, as well as many others, in common with the group.

Important in showing the close relationship between *S. zymogenes* and *S. fecalis* are what might be termed "tolerance tests" which bring out the greater vigor and resistance of the enterococcus group in comparison with the other streptococci. Sherman and Stark (1934) showed that among the characteristics which differentiate *S. fecalis* from *Streptococcus lactis* are the ability of the former to grow in the presence of a relatively high concentration of sodium chloride, and in media of more alkaline reaction. As opposed to *S. lactis*, *S. fecalis* grows vigorously in media containing 6.5 per cent sodium chloride, and in others having pH values of 9.6. The greater tolerance of sodium chloride and alkali by *S. fecalis* as compared with other streptococci has recently been confirmed by Chapman (1936). Our studies of *S. zymogenes* have shown that this organism also can grow in the presence of 6.5 per cent sodium chloride, and in alkaline media of pH 9.6.

The inhibitory action of medicinal methylene blue in dilute solution, introduced by Sherman and Albus (1918), has long been used as a differential test with certain groups of the streptococci. If the concentration of this dye is increased to 0.1 per cent in

sterile skimmed milk, the better-known streptococci are inhibited with the exceptions of the members of the "enterococcus" and "lactic" groups, which types are not inhibited by somewhat greater concentrations. *S. zymogenes* grows in the presence of this concentration of methylene blue.

Without laboring the point further, this brief discussion should make clearer the fact that *S. zymogenes* belongs in the enterococcus group and is closely related to the central species, *S. fecalis*. Proteolytic variants of the *S. fecalis* group have been noted since the time of Andrewes and Horder (1906), and the general relationship of *S. zymogenes* to that species has of course been recognized by most of the recent investigators (Sherman and Stark, 1931; Torrey and Montu, 1934; Elser and Thomas, 1936).

PROTEOLYTIC PROPERTIES

Although *Streptococcus zymogenes* has always been described as liquefying gelatin and digesting the casein of milk, we have repeatedly obtained non-liquefying strains of this hemolytic organism from the stools of the individual who has served as our constant source of the organism. The strains which fail to liquefy gelatin likewise show no visible evidence of peptonization of casein in milk cultures. Many cultures, other than those of the small collection which has been subjected to extended study, have been tested for this property. When isolations are made, sometimes only liquefying types are obtained, more rarely only nonliquefiers are found, while on some occasions both types are isolated. The proteolytic form has been, on the whole, the prevailing type obtained from this particular source. The nonproteolytic form is identical in all other features studied with the typical form. Elser and Thomas (1936) have reported that two cultures obtained from the American Type Culture Collection as hemolytic varieties of *S. fecalis* agreed well in cultural and biochemical properties with their cultures of *S. zymogenes*.

Variability in the liquefaction of gelatin by strains within the species is well known in the *Proteus* and *Aerobacter* groups. Among bacteria generally, the inability to liquefy gelatin is certainly a very constant characteristic in most non-proteolytic species; in proteolytic species, on the other hand, it is probable

that non-liquefying strains are not rare. Certainly, we know this to be the the case in some groups. This, after all, is in keeping with the generally recognized fact that mutation in the direction of loss of characters is more common than those mutations which add characters (though in the present case it should be admitted that we have no information concerning whether the supposed loss of gelatin liquefying power is a mutation or a gradual change).

In suggesting that apparently non-proteolytic strains of *S. zymogenes* be recognized, we are aware of the thin and shaky boundaries which are left to separate the supposed species in the enterococcus group: *S. liquefaciens* differs from *S. fecalis* only in proteolytic properties; *S. zymogenes* differs from *S. liquefaciens* in being hemolytic; non-proteolytic strains of *S. zymogenes* differ from *S. fecalis* in being hemolytic.

HEMOLYTIC PROPERTIES

From the particular source used by us to obtain this organism, we have isolated only hemolytic strains of *S. zymogenes*. Among these cultures there has been no variation observed from a distinct beta reaction. On the other hand, we have also studied the non-hemolytic *S. liquefaciens* obtained from other fecal sources and from milk. As has been previously shown, *S. zymogenes* and *S. liquefaciens* appear to be identical except in their actions on blood (Frobisher and Denny, 1928; Sherman and Stark, 1931).

Frobisher and Denny studied five cultures of *S. zymogenes*, three of which were of the beta type, one each of the alpha and gamma types, together with one culture of *S. liquefaciens* which gave the gamma reaction. Although most workers have considered *S. zymogenes* as being hemolytic, Elser and Thomas (1936), whose experience is more extensive, have found only alpha types among their organisms recovered in pure culture from the blood in subacute cases of endocarditis. There is of course nothing sacred about the action of streptococci on blood. The literature records many cases of the loss of hemolytic power, including observations on single-cell cultures (Grinnell, 1928) and Lancefield strain-specific cultures (Lancefield, 1934).

We would be the last to argue for the retention of *S. liquefaciens*

as a separate species for the non-hemolytic type. In the present state of our knowledge there would be little force to such an argument other than that of convenience. The work of Elser and Thomas (1936) gives strong support to the view that *S. zymogenes* should be considered as giving diverse reactions on blood.

FERMENTATION REACTIONS

Individual strains of *Streptococcus fecalis* are known to be extremely diverse in the results yielded with the fermentation tests; so much so, in fact, that the species has only in recent years been fairly clearly defined as a result of the advent of other and more fundamental tests. One of the reasons for considering the gelatin-liquefying types as species, distinct from *S. fecalis*, was that they appeared to ferment more constantly the resistant substances such as raffinose and glycerol. This was true in the small collections of *S. liquefaciens* studied by Orla-Jensen (1919), Hucker (1928), and Sherman and Stark (1931); and also for the cultures of *S. zymogenes* reported by Sherman and Stark. It should be remembered, however, that the total number of these types which have been subjected to detailed study is too small to reveal the less common variants. Although greater fermentative activity is indicated in the proteolytic types, it is probable that more extensive collections would reveal greater diversity. Our somewhat more extended study of *S. zymogenes* indicates that this is the case.

All of the twelve cultures of *S. zymogenes* studied by Sherman and Stark fermented arabinose and raffinose, but these results have not been borne out with larger numbers, non-fermenting strains being of common occurrence. Also, we have encountered one non-liquefying strain which failed to ferment glycerol. As these organisms ferment so many of the more resistant test substances, it is of interest to note that we have not found a strain of *S. zymogenes* or *S. liquefaciens* which fermented inulin, although in a collection of over 400 cultures of *S. fecalis* (Sherman, Mauer and Stark, 1937) a few were found to ferment this substance; a rather plain suggestion that an insufficient number of the liquefying types have been studied in order to bring to light the less

frequent variants. In view of the much more extensive available knowledge of *S. fecalis*, it is also logical to expect an occasional strain of *S. zymogenes* which does not ferment mannitol, though such a type has not been obtained among the limited number of cultures studied.

THE STATUS OF STREPTOCOCCUS ZYMOGENES AS A SPECIES

From what has been shown about the properties and the apparent variability of some of the characteristics of the organisms of this general type, there would appear to be much common sense in the suggestion that proteolytic or hemolytic enterococci be considered simply as varieties of *Streptococcus fecalis*. From this point of view, the general group might be considered as consisting of the one species with its several varieties:

Streptococcus fecalis (hemolysis −, proteolysis −)
S. fecalis var. hemolyticus (hemolysis +, proteolysis −)
S. fecalis var. liquefaciens (hemolysis −, proteolysis +)
S. fecalis var. zymogenes (hemolysis +, proteolysis +)

For practical purposes, however, it is likely that bacteriologists will prefer to classify proteolytic, and hemolytic, streptococci of this group as separate species, and almost certainly when these two characteristics are combined in the same organism. Under any of these three circumstances, *S. zymogenes* seems likely to survive as a species name. If proteolysis is regarded as fundamental and hemolysis as variable, *S. zymogenes* would have priority over *S. liquefaciens* (Orla-Jensen) as the name for the group. If hemolysis is considered the proper point of departure, with proteolytic activity variable, *S. zymogenes* would again seem to be the proper designation. It is the only name now in use which could appropriately be applied to an "enterococcus" defined as both hemolytic and proteolytic.

As lines between species are now generally drawn in bacterial taxonomy, there are ample precedents as well as convenience in recognizing the proteolytic and hemolytic *S. zymogenes* as a quite distinct species type from the non-proteolytic and non-hemolytic *S. fecalis*. This would leave as connecting links those types

which may be proteolytic but not hemolytic (*S. liquefaciens*) or hemolytic but not proteolytic.

The status of these "connecting links" as varieties or species may perhaps be best left to the taxonomists. We feel, however, that taxonomic zeal should not outrun our knowledge of bacteria with the resulting creation of species based upon slight, and perhaps insignificant, differences. Although we favor a conservative attitude in the recognition of species, attention to what now appear minor differences is apt to stimulate laboratory research which will further fortify or show the artificiality of such distinctions. It should be remembered that only twenty years ago papers were appearing which purported to show the essential identity of such widely different species as *S. lactis* and *S. pyogenes*; and even today publications appear that claim or imply the identity of the fairly clearly differentiated *S. lactis* and *S. fecalis*. One need look no further than to that inchoate jumble of organisms that still go under the label of "*Streptococcus viridans*" to appreciate the need for more detailed studies of the streptococci.

In recommending that *S. zymogenes* be adopted as the most appropriate name for this group of streptococci, a few additional words may be in order. MacCallum and Hastings gave a remarkably clear description of their organism considering the period in which the work was done, identified it with fecal matter, and also as an occasional invader of the human body. The name has since had a fairly continuous use by workers who have identified similar organisms from stools and clinical sources. The fact that MacCallum and Hastings used *Micrococcus* as the generic designation would no more invalidate their species name than did Lister's use of *Bacterium lactis* for the milk-souring streptococcus which now almost universally carries the species name which he applied to it.

The older bacteriological literature has references to many streptococci which were said to liquefy gelatin. These, such as the *S. liquefaciens* of Sternberg (1892), could not be now identified. The organism which is sometimes given priority is the *Streptococcus coli-gracilis* of Escherich, frequently designated in

later manuals under the binomial, *S. gracilis*. We have not been able to consult the original publication of Escherich, but his organism is described in rather full detail in a number of the early volumes on bacteriology. If the reproduced descriptions are faithful ones, it can be said with considerable assurance that Escherich's organism was not the same as *S. zymogenes*.

THE CHARACTERISTICS OF STREPTOCOCCUS ZYMOGENES

The following description is of the typical hemolytic and proteolytic form since we advocate, for the present, the recognition of the non-proteolytic and the non-hemolytic types as varieties of the species. Except for their actions either on blood or on gelatin and milk, these types do not differ in any known way from the typical form.

Morphological and cultural characteristics

The characteristic grouping is in pairs, though chains also occur. Blood is hemolyzed; gelatin is liquefied; milk is acidulated and curdled, the curd also being peptonized.

Significant physiological characteristics

Growth takes place at 10°C.; at 45°C.; in the presence of 6.5 per cent sodium chloride; in alkaline media with pH values of 9.6; and in relatively strong solutions of methylene blue (0.1 per cent in skimmed milk). The organism survives heating at 62.8°C. for thirty minutes in skimmed milk.

It is characterized by a strong reducing action, litmus in milk cultures being completely reduced before the milk is curdled. In glucose broth a final pH of 4.4 to 4.0 is attained; ammonia is produced in 4 per cent peptone; esculin is hydrolyzed; starch is not hydrolyzed; sodium hippurate may or may not be hydrolyzed. Acetyl-methyl-carbinol is produced in milk cultures.

Fermentation reactions

Glucose, maltose, lactose, sucrose, mannitol, and salicin are fermented; glycerol is usually fermented (25 of 26 cultures); arabinose and raffinose may or may not be fermented; inulin is not fermented.

Varieties

(1) A hemolytic but apparently non-proteolytic type, not liquefying gelatin and causing no visible peptonization in milk.

(2) A proteolytic but non-hemolytic type.

THE LANCEFIELD GROUP D HEMOLYTIC STREPTOCOCCUS

Students of the streptococci have doubtless been generally impressed with the fact that the Lancefield (1933) Group D type of hemolytic streptococcus appears to be very different from the better known types of human and animal pathogens, represented by her Groups A, B and C. From the limited physiological data at hand, this fact is plainly indicated by the greater tolerance of the D type to bile, methylene blue, and heat, and its ability to produce, in general, a lower final pH in glucose broth. The fact that the D type has been referred to as a "dairy organism," together with our own knowledge that *Streptococcus zymogenes* occurs commonly in pasteurized milk, led naturally to the suspicion that *S. zymogenes* and the Group D type are the same. That this general relationship was clearly recognized by Dr. Lancefield is shown by the statement: "These (Group D) streptococci resemble the group of enterococci or *S. faecalis* more than *S. pyogenes* because they are characteristically lanceolate, grow in moist luxuriant colonies, and a proportion resist heat at 60°C. for 30 minutes" (Lancefield and Hare, 1935).

Through the generous coöperation of Dr. Lancefield, who sent us four of her Group D strains, the identity of this type with *S. zymogenes* appears to be established. Two of these cultures (C6 and H108) proved to be typical gelatin-liquefying strains of *S. zymogenes*. One strain (H69D5) agreed in all characteristics with those cultures which we have herein described as the non-proteolytic variety of this species, differing from the type species in its actions on gelatin and casein.

The fact that the Lancefield Group D includes both liquefying and non-liquefying types adds much weight to our contention that a non-proteolytic variety of *S. zymogenes* be recognized.

The fourth culture (C3) obtained from Dr. Lancefield is of

especial interest. This is a non-liquefying strain which could be properly considered an "atypical *S. zymogenes*," but which could not be classified with any other as yet known species of hemolytic streptococcus. This strain is atypical in a number of respects and lacks the general vigor and tolerance which is characteristic of *S. zymogenes*: it might perhaps best be described as "physiologically attenuated." Of particular importance in this connection is the personally communicated information from Dr. Lancefield that this strain represents a distinct serological type, within Group D, from any of the other three cultures studied by us. The information obtained about this "atypical" culture, with very different modes of attack, is another illustration of the fact that physiological and immuno-chemical data can generally be integrated and harmonized if the cultural studies are sufficiently comprehensive.

For the further elucidation of the large number of ill-defined, non-pathogenic, hemolytic streptococci, it would be of much value to know whether the Lancefield Group D is species-specific for *S. zymogenes* or if this group also includes other "hemolytic enterococci," such as *Streptococcus durans* and lesser known types.

SUMMARY

A detailed description is given of *Streptococcus zymogenes*, based upon a more varied set of characteristics than has heretofore been used for this purpose.

Streptococcus zymogenes is clearly shown to be a member of the "enterococcus group," closely related to *Streptococcus fecalis*.

It is recommended that the name *Streptococcus zymogenes* be retained for enterococcus-like streptococci which are hemolytic and proteolytic, and that non-proteolytic or non-hemolytic strains be regarded, for the present, as varieties.

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VARIATIONS IN THE FILTRABILITY OF DIFFERENT RACES OF BACTERIOPHAGE[1]

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Received for publication November 27, 1936

It is generally considered that the size of the particles of different bacteriophage races is the same although there are wide differences in estimates of the actual dimensions. Though no attempt has been made to determine the exact size of phage corpuscles, we have found that considerable variation in filtrability exists. Our studies indicate that some races of bacteriophage readily pass certain types of filters, while others under apparently similar conditions are retained.

In the present experiments, races of phage lytic for *Escherichia coli*, *Streptococcus haemolyticus*, *Streptococcus faecalis*, and numerous staphylococci were employed. The samples possessed a titer of at least 10^{-8} as measured by the serial dilution method. They were grown on organisms obtained from human patients and were freshly prepared. The media was 1 per cent "Savita." With phenol red as the indicator, the pH of the different samples was between 7.2 and 7.4. Summarizing most of the experimental data to 1936, Krueger (1936) concluded that between pH 3.4 and 12.0, with only infrequent exceptions, phages bear a negative electrical charge.

The filter candles used were Chamberland L_3, L_5, and L_7 and the Coors. They are all of the siliceous type. According to Rivers (1928), their electrical charge is that of the silicates, the filter pore walls being charged negatively. The pore diameters of the Chamberland L_3 average 2.7 μ. No figures could be obtained for the Coors, but judging by the speed of filtration,

[1] Prepared with the aid of a grant from the Isaac Kaufmann Foundation.

they were much the finest, the Chamberland L_7, L_5, and L_3 increasing respectively in coarseness. Five Coors candles were used and about 15 Chamberland, with at least three of each labeled porosity. Though not new, the candles had been used only in the preparation of bacteriophage for clinical purposes, following the author's technique which permits the repeated use of filters and rapidly yields a product of high titer. A minimum of three filtrations of each specimen in two candles of each type and porosity was done, but there were a large number of similar filtrations performed without formal study. The same candle after sterilization was frequently used as a check upon itself. In addition, the essential features of the experiment were repeated 18 months later with identical results. During the past three years, using many cultures from different sources, there has been no significant variance from the original findings.

PROCEDURE

A specific bacteriophage was allowed to act on a young broth culture of the sensitive organism until lysis occurred. Portions of the culture were then passed through the various candles under similar conditions of negative pressure and the filtrates tested individually for the lytic agent. The criteria accepted for its presence were (1) plaque formation on solid mediums and (2) lysis of young broth cultures of the homologous organisms. Samples of phage which had not been in recent contact with susceptible organisms were filtered and tested similarily. Also, mixtures of the four phage races and various combinations of them were submitted to the same tests.

FINDINGS

In these tests races of phage lytic for *Staphylococcus aureus* and *Streptococcus faecalis* readily passed the L_3, L_5, and L_7 Chamberland candles but did not pass the Coors. Races lytic for *Streptococcus haemolyticus* and *Escherichia coli*, however, were able to pass both types. Mixtures of the four races passed all the Chamberland candles but only the components lytic for *Streptococcus haemolyticus* and *Escherichia coli* passed the Coors. Whether a

race of phage had been filtered previously or whether it had just completed the lysis of a young culture did not appear to modify the conditions of filtrability. No alterations in filtrability were noted 10, 20, 30, 60, and 90 days after the preparation of the filtrate. Storage at refrigerator, room, and 37.5°C. temperatures did not influence filtrability.

Variation in filtrability among different strains of the same race of bacteriophage was observed with *Staphylococcus aureus* obtained from three cases of human chronic osteomyelitis. Staphylococcus phage completely lysed these cultures but the filtrates therefrom were inactive. The same filters, however, both before and after these tests permitted other staphylococcus phages to pass. Moreover when some of the unfiltered lysate was added to susceptible cultures of other strains typical lysis occurred, and filtrates from such lysates were propagable. No morphological, colonial, nor tinctorial peculiarities were observed in those cultures whose lysates failed to yield active filtrates.

DISCUSSION

It might be suggested that the reported variations in filtrability are due to differences in the size of the bacteriophage particles, although it is recognized that the pore spaces of any candle used are larger than even the greatest estimated size of a phage corpuscle. Our findings, however, are consistent with the observations of Elford and Andrewes (1932) who, using graded collodion membranes (gradocol) and checking by diffusion through Jena sintered glass discs, concluded that Coli phage particles had a diameter of about 30 $\mu\mu$, while Staphylococcus phage particles ranged between 50 and 75 $\mu\mu$. In further corroboration we observed that the more easily filtrable (smaller?) Coli phage formed plaques 8 to 10 times broader than the less filtrable (larger?) Staphylococcus phage.

It is furthermore difficult to explain the variations in filtrability on the basis of differences in the charges on the candles, since all were of the siliceous type. The high concentration of the phages employed also lessens the possibility that failure to pass the finer filters was due to their greater absorptive power,

while the ability of certain components of a mixture to pass while others were retained negates the criticism that some substance which aided or impeded filtrability had been set free in the medium. These observed variations in filtrability may explain the inability of some workers to reproduce the phenomena of bacteriophagy with supposedly potent phage supplied by other laboratories.

SUMMARY

Of four races of bacteriophage lytic for *Escherichia coli, Streptococcus haemolyticus, Streptococcus faecalis,* and many staphylococci, all of which passed Chamberland candles of various porosities, only the first two were also able to pass through the Coors—a much finer filter. Cultures of *Staphylococcus aureus* obtained from three cases of osteomyelitis and lysed by staphylococcus phage, gave a lysate that was withheld by filter candles capable of passing other phages for staphylococci. The variations in filtrability were not altered by the age of the filtrates, by storage temperatures, by contacts with the homologous cultures, nor by mixtures with definitely filtrable races of phage. Although not proved by the filtration experiments it is suggested that the observed variations in filtrability are due to differences in the size of bacteriophage races.

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BACTERIAL TYPE TRANSFORMATION

IV. Micrococcus Tetragenus Infection[1]

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Received for publication December 1, 1936

In recent years it has become obvious that the problem of bacterial variation is vastly more complex than was at first believed. It appears that bacteria possess several methods of variation or ability to alter their morphologic and biologic nature. One mode of variation concerns the widely studied culture phases commonly regarded as the mucoid (M), smooth (S) and rough (R) phases of a given type, one involves the apparent change from coccus to bacillary form or *vice versa*, another the development of translucent and lytic forms, and still another concerns actual type transformation. All four modes of variation were observed in studies on a strain of *Micrococcus tetragenus* (Reimann, 1936, a, b, c, d.)

The possibility of type transformation has been suggested for years, especially in regard to the meningococcus and gonococcus (Atkin, 1925) and among the colon-typhoid group of bacilli. The question has been of especial interest in regard to the relationship of small-pox virus to vaccinia virus, and between the so-called "street" and "fixed" virus of rabies. Changes involving the conversion of pneumococci into streptococci have not been generally recognized although the recent studies of Paul (1934) show that certain R dissociants of pneumococci are indistinguishable from certain strains of *Streptococcus viridans*. It

[1] This work was done in the Department of Medicine, at the University of Minnesota and was aided by a grant from the Graduate School Fund.

is now known that type transformation can be induced artificially among pneumococci (Griffith 1928, Reimann 1929). It may occur spontaneously, as appears to be the case concerning murine and epidemic typhus (Mooser, 1934) and the rabbit papilloma virus of Andrewes and Shope (1936). Among most species of bacteria, type transformation is difficult to detect on culture media without laborious study since the separate types are seldom distinguishable by differences in colony structure or color. In the case of *M. tetragenus*, the problem was simplified by the distinctive color of many of the types and the ease with which specific agglutinating serum could be prepared with the respective types.

In previous papers, the isolation and biologic characteristics of ten variant forms of a strain of *M. tetragenus* were described (Reimann, 1936 a, b). The existence of three mucoid forms of the same organism and the absence of typical rough forms made it impossible at the time to correlate the variants observed with the orderly pattern of microbic dissociation found among other bacteria. The key to the problem was not provided until many months later when the appearance of a rough pink form in an aged broth culture led to a grouping of the pink forms at hand into a single type with mucoid, smooth and rough culture phases. This procedure suggested the rearrangement of the other observed variant forms into separate types with respective culture phases as follows (Reimann, 1935c),

Mucoid-yellow	Mucoid-white	Mucoid-pink		
Smooth-yellow	Smooth-white	Smooth-pink	Smooth-pink-yellow	Smooth-brown
		Rough-pink		

Under this arrangement the existence of the mucoid-pink-yellow, mucoid-brown, and the rough forms of the yellow, white, pink-yellow, and brown types was predicted. A description of the subsequent detection, isolation, and study of most of the predicted forms is embodied in this paper.

ISOLATION OF THE PREDICTED MUCOID PHASES

Previous success in obtaining mucoid forms in 100 cc. broth culture flasks aged for months at room temperature of about 25°C.

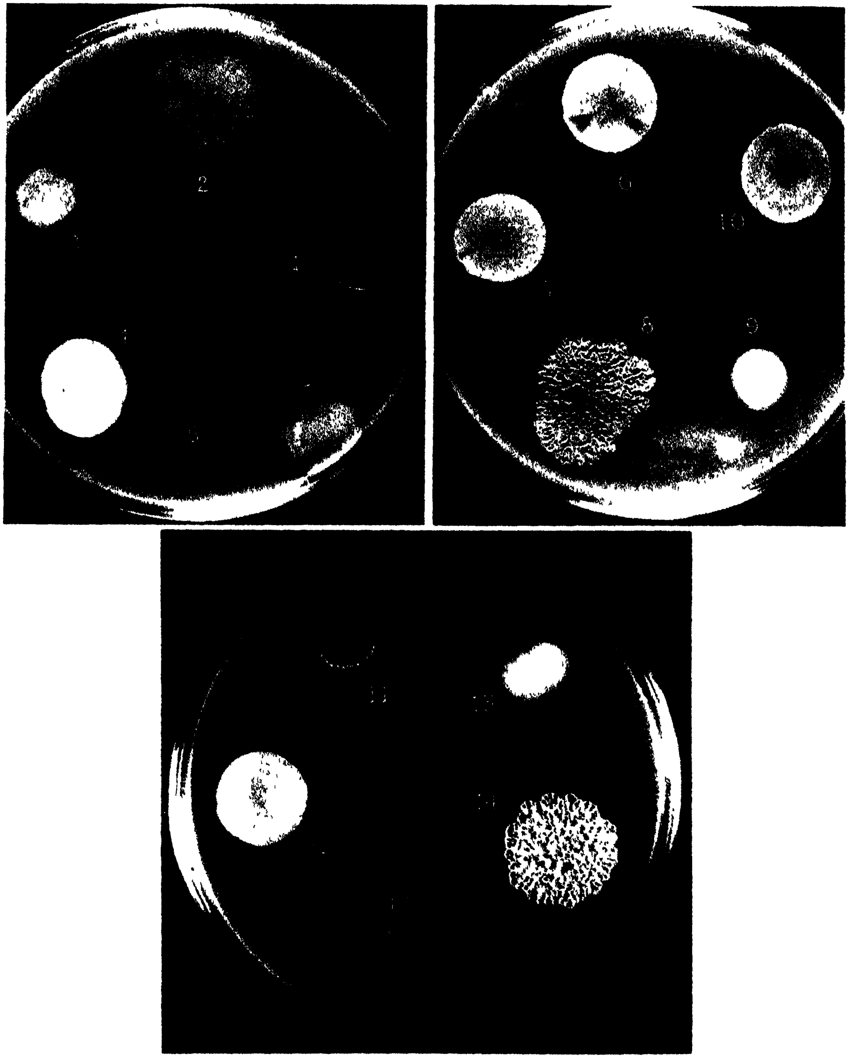

FIG. 1 PHOTOGRAPHS, SLIGHTLY REDUCED, OF POINT COLONIES ON DEEP AGAR PLATES SEEDED SIMULTANEOUSLY WITH 15 VARIANT FORMS AND INCUBATED FOR 17 DAYS AT 25°C.

(*1*) Mucoid-yellow, (*2*) Smooth-yellow, (*3*) Mucoid-white, (*4*) Smooth-white, (*5*) Translucent, (the extra colony is a contaminant) (*6*) Mucoid-pink, (*7*) Smooth-pink, (*8*) Rough-pink, (*9*) Mucoid-pink-yellow, (*10*) Smooth-pink-yellow, (*11*) Rough-pink-yellow, (*12*) Mucoid-brown, (*13*) Smooth-brown, (*14*) Rough-brown and (*15*) Bacillary colony. The rough yellow colony is not shown, save for the pigment, it resembles that of the rough pink colony (*8*). Sector formation may be noted in the smooth yellow (*2*), mucoid pink (*6*) and smooth pink (*7*) colonies.

led to the continued use of this technique. On several occasions one mucoid type gave rise to another mucoid type after aging on deep agar plates.

A 100 cc. plain-broth-culture of the S-pink-yellow type aged for one year at 25°C. was plated on agar. A few colonies of the original pink-yellow form appeared but the growth consisted chiefly of thick, pearly, translucent, mucinous, semifluid colonies which tended to run like the other mucoid forms previously described when the plate was tilted (colony 9, fig. 1). Although these colonies were not visibly pigmented, the term "mucoid-pink-yellow" was applied for simplicity. Cocci of the mucoid colony were small, mostly in diplo- and tetrad arrangement and often surrounded by an amorphous, pink-stained material presumably capsular in origin.

The mucoid-brown form was isolated from a 9-month-old broth culture of the S-brown form, but in this case dissociation seemed complete since none of the original S forms grew when plated on agar. The mucoid-brown colonies were thick, cream-colored and mucinous, tougher and less fluid than mucoid colonies of the other types (colony 12, fig. 1). The colonies often adhered firmly to the agar; the cocci resembled those of the mucoid pink-yellow form morphologically.

ISOLATION OF THE R PHASES

The rough-pink form was detected after an 8-month-old broth culture of the smooth-pink form was plated. During the first week the colonies all resembled the S-pink form but gradually a few became dryer, deeply crinkled (colony 8, fig. 1), and could be picked up *in toto*. Later the rough colonies became firmly adherent to the agar. The cocci resembled those of the original S form but old cultures could not be emulsified in physiological salt solution. When cultures of the R form were inoculated into subcutaneous agar foci in mice, numbers of S form colonies appeared among the R forms on agar plates when cultures were made after a week, indicating that reversion had occurred.

The rough-pink-yellow phase was encountered after inoculating a test tube of broth with a 9-month-old broth culture, and plat-

ing on agar after 7 days at 25°C. On the plate grew the usual S-pink-yellow form, but after 5 days, many of the colonies developed a coarsely granular surface unlike the wrinkled and folded surfaces of the R-pink colonies (colony 11, fig. 1). The R forms bred true on subculture but one point colony seeded with the R-pink-yellow form developed into a smooth-pink-yellow colony in which several rough sectors later appeared. The cocci resembled those of the S form. Old cultures agglutinated spontaneously in normal salt solution.

The rough-brown colonies appeared after a 9-month-old flask culture was transferred once in a test tube of broth and plated on agar. The growth consisted chiefly of mucoid-brown forms with here and there thin, cream-colored, crumbly, friable, deeply crinkled colonies with scalloped edges. Although the cocci like those of the mucoid-brown were not visibly pigmented, the colonies they produced were called "rough-brown" to indicate their relationship with the S-brown forms. Point colonies of the R-brown form were large and spreading (colony 14, fig. 1). The surface when examined with a hand lens was marked by folded and intertwined deep, waxy wrinkles and had a scalloped margin, resembling in effect a lace doily. The cocci were uncharacteristic and were metachromatic like those of the other forms.

The rough-yellow was encountered after growing the smooth-yellow form in a 100 cc. flask of broth for 16 months and also after 4 months in "starvation" media composed of broth diluted from 16 to 64 times with distilled water. After seeding the cultures on agar all colonies appeared to be the same at first, but after a week numbers of colonies became drier and developed deep radial striae while others gradually developed deep waxy wrinkles or lumpy surfaces with irregular margins. The rough colonies were thin, tough, stringy and adhered to the agar. Reversion to the S form colony frequently occurred. The cocci were uncharacteristic. Numerous white and translucent colonies appeared on plates seeded from the aged cultures.

Rough-white forms were not encountered although several methods known to favor dissociation were employed. Growth of the smooth-white form after several months at 25°C. in 100 cc.

flasks of "starvation" media composed of broth diluted with distilled water in proportions of 1:32 and 1:64 respectively, or after rapid transfer in test tubes containing 1 part of freshly prepared specific immune serum and 9 parts of broth diluted 1:8 with

TABLE 1

Biologic characteristics of five types and their respective culture phases derived from a strain of M. tetragenus

	ACID IN					GELATIN LIQUIFIED AFTER 2 WEEKS AT 25°C.	GROWTH AT				RESISTANT TO ULTRAVIOLET LIGHT 1 MIN.
	Glucose	Sucrose	Maltose	Lactose	Mannitol		10°C.	42°C.	pH 5.5	pH 10	
Mucoid-yellow	−	−	−	−	−	+	−	−	−	+	
Smooth-yellow	−	−	−	−	−	+	+	−	−	+	+
Rough-yellow	−	−	−	−	−	+	+	−	−	+	
Mucoid-white	−	−	−	−	−	+	−	+	−	+	
Smooth-white	−	−	−	−	−	−	−	+	+	−	−
Mucoid-pink	−	−	−	−	−	+	−	−	+	+	+
Smooth-pink	±	−	−	−	−	−	+	−	−	+	+
Rough-pink	±	−	−	−	−	+	+	−	−	+	+
Mucoid-pink-yellow	−	−	−	−	−	+	−	+	−	+	−
Smooth-pink-yellow	±	−	−	−	−	−	+	−	−	+	−
Rough-pink-yellow	±	−	±	−	−	−	+	−	−	+	+
Mucoid-brown	±	±	±	±	−	+	+	−	+	+	−
Smooth-brown	−	−	−	−	±	−	−	+	+	−	−
Rough-brown	±	±	±	−	−	+	−	+	+	+	+
	+ = pink ± faint change − = no change After 48 hours at 37°C. and 3 days at 25°C.						+ = growth − = no growth				

sterile water, or after aging 16 months in a broth flask, was usually composed of the mucoid and smooth forms in varying proportions. On a number of occasions a few or many translucent forms and an occasional yellow form developed.

BIOLOGIC CHARACTERISTICS OF THE TYPES AND THEIR CULTURE PHASES

Certain biologic reactions of the various forms are summarized in table 1. Gelatin liquefication and sugar fermentation tests were not constant. The average of many tests showed that the yellow and white types, the M-pink, and the M-pink-yellow usually did not ferment any of the sugars. Only the S-brown form fermented mannitol. Gelatin was usually liquefied by all the mucoid forms and all the rough forms except R-pink-yellow, and by none of the smooth forms except the S-yellow. The yellow, pink and pink-yellow types grew better at lower temperature and in more alkaline media than the white or brown types. The varied behavior of growth and resistance to adverse circumstances will be discussed in detail in the following paper. In general the biologic reactions of the M forms differed more from their respective S and R forms than from each other.

SEROLOGY OF THE CULTURE PHASES

Immune sera were prepared and agglutination tests were made in the usual manner. The results of the tests are shown in table 2.

The anti-mucoid-yellow serum agglutinated its own form in a titer of 1:160, but not the S-yellow form from which it was derived. The anti-smooth-yellow serum failed to clump the mucoid-yellow to a significant titer but clumped smooth yellow at 1:160. The anti-mucoid-white and the anti-smooth-white sera were, however, serologically similar and caused mutual clumping of the respective cocci.

The anti-mucoid-pink serum, like the anti-mucoid-yellow, failed to clump its own S or R forms, nor were mucoid-pink cocci clumped in S-pink or R-pink antisera. Both S-pink and R-pink antisera agglutinated the S- and R-pink-yellow cocci (table 3) with which they are serologically related as previously shown. The mucoid-pink-yellow organisms likewise were not clumped by antiserum prepared with the S-pink-yellow from which they were derived or with R-pink-yellow antiserum. Both S- and R-pink-yellow antiserum clumped smooth-pink forms and to a lesser

TABLE 2

Agglutination reactions of certain variant forms and antisera prepared with these forms

		DILUTION OF SERUM					
		1:10	1:20	1:40	1:80	1:160	1:320
Anti-mucoid-yellow serum	cocci M Y	+++	+++	+++	+++	+	−
	S Y	−	−	−	−	−	−
Anti-smooth-yellow serum	cocci M Y	+	+	−	−	−	−
	S Y	−	+	+	+	+	−
Anti-mucoid-white serum	cocci M W	++	++	+++	++	+	+
	S W	++	++	+	+	+	−
Anti-smooth-white serum	cocci M W	+++	+++	+++	++	++	+
	S W	++	+++	++	++	+	−
Anti-mucoid-pink serum	cocci M P	+++	++	+	+	−	−
	S P	−	−	−	−	−	−
	R P	−	−	−	−	−	−
Anti-smooth-pink serum	cocci M P	−	−	−	−	−	−
	S P	+	++	++	+	+	−
	R P	+	++	+	+	−	−
Anti-rough-pink serum	cocci M P	−	−	−	−	−	−
	S P	+	++	++	++	+	+
	R P	++	++	++	++	+	+
Anti-smooth-pink-yellow serum	cocci M P Y	−	−	−	−	−	−
	S P Y	++++	++++	++	++	+	−
	R P Y	+	+	+	+	+	−
Anti-rough-pink-yellow serum	cocci M P Y	−	−	−	−	−	−
	S P Y	++++	++++	++	+	−	−
	R P Y	+	+	+	+	+	+
Anti-mucoid-brown serum	cocci M B	+++++	++++	−	−	−	−
	S B	++	+	+			
	R B	++++	++	++	+	+	−
Anti-smooth-brown serum	cocci M B	++++	++++	++	+	−	−
	S B	++	+	+	−	−	−
	R B	++++	++	+	−	−	−
Anti-rough-brown serum	cocci M B	++++	++++	+	+	−	−
	S B	++	+	+	−	−	−
	R B	++++	++	++	+		−

degree smooth-yellow cocci (table 3) as shown in a previous paper (Reimann, 1936b).

The M-, S-, and R-brown phases were similar serologically, each anti-serum caused agglutination of its own and both of the other phases. Difficulty in reading results with S-brown organisms because of spontaneous clumping frequently arose.

Although evidence of cross immunity between the mucoid-yellow, mucoid-pink and mucoid-white types was noted in study III, the mucoid-pink-yellow and mucoid-brown were not related serologically to other mucoid forms. The absence of demonstrable immunologic relationship between the mucoid forms of the yellow, pink and pink-yellow types with their respective S and R is difficult to understand. The pigment of the mucoid-pink form

TABLE 3

Cross-immune reactions between the pink and pink-yellow types

	SERUM	1:10	1:20	1:40	1:80	1:160	1:320
Smooth-pink-yellow cocci	Anti-mucoid-pink	−	−	−	−	−	−
	Anti-smooth-pink	+	++	++	+	−	−
	Anti-rough-pink	+	+	++	++	+	+
Smooth-pink cocci	Anti-smooth-pink-yellow	+	++	++	++	+	+
	Anti-rough-pink-yellow	++	+++	+	+	+	−

also differed from that of its S and R forms as will be shown in a subsequent paper.

EVIDENCE OF THE EXISTENCE OF ADDITIONAL TYPES

During the course of observation and examination of hundreds of agar plate cultures of the various types and phases, evidence of change of type was occasionally noted. In several instances the newly appeared cocci were tested to determine whether they were identical with the previously isolated forms which they resembled. For example, on one occasion, the smooth-yellow form gave rise to white colonies after 8 months incubation in broth at 25°C. The new smooth-white forms were not, however, agglutinated by the stock anti-smooth-white serum. On another

occasion the mucoid-pink form gave rise to a mucoid-yellow and a mucoid-white form during growth in an agar plate. The mucoid-yellow cocci were clumped by the stock mucoid-yellow antiserum, but the mucoid-white forms were heterologous with the stock anti-mucoid-white serum. Other similar occurrences were noted.

Evidence of the existence of different serologic types among organisms producing colonies morphologically identical as mentioned in the preceding paragraph suggested the existence of other specific types in addition to those under investigation. Other observations also supported this possibility. On one occasion an aged broth culture of the mucoid-pink type gave rise to three different forms of mucoid-pink colonies; one was light pink, another dark pink and the third had a brownish hue. Point colonies of each form reproduced similar forms for several transfers.

DISCUSSION

The detection and isolation of the numerous types obtained from a single strain of *M. tetragenus* together with various culture phases of these types has been the result of over two years of study. Hundreds of plate cultures were examined, and only occasionally among many similar colonies would one or more colonies, daughter colonies or sectors of colonies of different appearance be evident, which then required isolation and study. Aging for weeks on agar surfaces in spreads or in point colonies, or for months in 100 cc. flasks of broth at 25°C. were the simplest methods suited to induce type and phase variation. Type variation also occurred *in vivo*. The possibility of contamination was always in mind. Only those colonies which appeared as daughter papillae or as sectors or adjacent colonies on plates, and those which appeared with the original forms in broth culture made under rigid technic were isolated for study. The evidence of direct or indirect serologic relationship, biologic similarities, the frequent appearance of translucent colonies among many of the types and their culture phases, and occasional reversion to the forms from which they were derived warranted the inclusion of the numerous forms studied in one large pattern of variation.

The cocci composing the various types and forms of colonies were generally indistinguishable from one another in stained slide preparations. As a rule those of the yellow forms more often assumed a tetrad arrangement, and those of the translucent form were almost always much smaller and more variable in size than the others. The rest were usually grouped in irregular clusters, occasionally in fours, in pairs or single. All were metachromatic and stained unevenly by Gram's method.

Variation appeared to be largely a chance phenomenon which could not be forced in any desired direction. The frequency of the appearance of variation was in proportion to the number of bacteria present in large cultures, especially when grown under the adverse conditions of faulty nutrition or other unfavorable environment. The simplest variation seemed to involve the S-white, S-yellow, and translucent forms as noted in paper I of these studies. In general the smooth forms gave rise to the mucoid and rough forms. On a few occasions one mucoid phase seemed to give rise directly to the mucoid-phase of a different type without passing through an intermediate stage. Somewhat similar observations have been made with the pneumococcus (Dawson and Warbasse, 1931). Mucoid phases were not seen to originate from the rough forms directly nor did mucoid phases produce rough forms. The rough forms occasionally reverted to the smooth phase of the same type. Aging of most of the mucoid forms especially of the yellow and white types, usually caused the appearance of translucent forms. The forms thus far included in these studies are arranged as follows:

Mucoid-yellow	Mucoid-white	Mucoid-pink	Mucoid-pink-yellow	Mucoid-brown
Smooth-yellow	Smooth-white	Smooth-pink	Smooth-pink-yellow	Smooth-brown
Rough-yellow		Rough-pink	Rough-pink-yellow	Rough-brown
		G-bacillary?		

The position of the translucent and translucent lytic forms in the pattern of variation is not understood nor is the presence of the bacillary form as discussed in study III. (Reimann, 1936b).

The arrangement of various types and culture-phases of types of a strain of *M. tetragenus* as proposed here is a new concept which I believe is supported by a number of facts. The existence of separate types is indicated by the stability of the forms

described, and by the evidence of their antigenic specificity and fairly constant biologic characteristics. The transformation of one type into another *in vivo* and *in vitro* finds analogy in the case of the pneumococcus as previously mentioned. It is reasonable, therefore, to believe that similar type interconvertibility may occur among other bacteria. The clinical and epidemiologic significance of the problem is obvious.

It is of interest to point out further similarities of type transformation and culture-phase changes between *M. tetragenus* and pneumococci. The pneumococcus, like *M. tetragenus*, is composed of specific types each apparently convertible into the other by special methods (Griffith 1928, Reimann 1929), and each type of the few thus studied, has culture-phases of M, S, and R forms. Similar comparison may be made with *Streptococcus hemolyticus* or with Friedländer's bacillus. The serologic relationship between Type II and Type V, and between Type III and Type VIII pneumococci is analogous perhaps with the antigenic similarity of the mucoid-yellow and mucoid-pink, and of the smooth-pink and smooth yellow and the smooth-pink-yellow types of *M. tetragenus.* By further analogy with the pneumococcus or streptococcus one may predict the existence of many more types of the strain of *M. tetragenus* under study. Evidence of the existence of more types than those studied has been mentioned.

It may also be recalled that in a previous paper (Reimann, 1936,b) a parallel specific immune relationship was shown to exist among the variant types of the strain under study and of the corresponding variant types of 3 of 11 strains from other sources. In other words, after the yellow or white types of my strain were found to be homologous with the yellow or white types of 3 other strains, cocci of the pink or brown variant types of these strains were also specifically agglutinated by antiserum prepared with my corresponding pink or brown types. None of the variant types of the 8 other strains was serologically identical with any of my antisera prepared with similarly pigmented types.

SUMMARY

A strain of *Micrococcus tetragenus* isolated from a patient was observed to possess at least 5 major variant forms which were re-

garded as specific types. There was evidence that more types exist. Each type was observed to have M, S, or R culture phases. One type may change into another spontaneously *in vivo* or *in vitro*. The significance of the translucent forms and bacillary forms frequently noted is unknown.

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THE SIGNIFICANCE OF BACTERIAL VARIATION

V. Micrococcus Tetragenus Infection[1]

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Received for publication December 1, 1936

A consideration of the biologic significance of microbic variation has led numerous observers to regard the phenomenon as essentially one of adaptation. Hadley (1927) who discussed the subject fully, suggested that microbic dissociation involves the partial or complete elimination by autolytic and transformatory processes of certain forms of a given organism when the changed conditions of environment become sufficiently unfavorable to a continuance of the same type of growth and is accompanied by the formation of new bacterial forms better qualified to perpetuate the stock in the changed environment. The transformation from one form to another appears to be a normal adaptive reaction made possible through the intervention of a special type of reproductive mechanism. Seiffert (1936), on the other hand, regards the matter differently. He believes that the appearance of bacterial "mutants" does not bear relationship to the environment, but is in proportion to the number of bacteria present. However, an environment unfavorable to one of two mutants will cause the elimination of one and permit growth of the other.

An opportunity to test these assumptions was conveniently provided by the numerous, easily recognizable variant forms of a strain of *Micrococcus tetragenus* described in previous papers (Reimann, 1936). Moreover, it was possible to test whether any one method of variation such as type transformation, culture-

[1] This work was done in the Department of Medicine at the University of Minnesota and was aided by a grant from the University of Minnesota Graduate School Fund.

phase dissociation or other modes of variation, was of more importance than another in the presumed process of adaptation. The five types and culture-phases of the respective types, and the translucent and bacillary forms isolated were therefore subjected to a variety of circumstances which are regarded as unfavorable to the growth and life of bacteria to determine whether or not striking variations of growth and resistance were demonstrable among the different forms.

GROWTH TESTS

Effect of temperature. Sets of agar plates which were marked off into five sectors, pie fashion, were seeded with the respective variant forms, sealed with adhesive tape to delay drying and incubated at 4, 10, 25, 37, 40, 42, 44 and 45°C. The plates were examined after 18 hours and at intervals thereafter. The average results of many tests are shown in table 1.

After 18 hours all forms grew about as well at 25°C. as at 37°. In general, after one week, the yellow, pink and pink-yellow types grew better toward a lower range of temperature than the other forms. Minor differences are shown in the table. For example, the mucoid forms failed to grow as well as the S and R forms at low temperature. The smooth-pink and rough-pink forms grew at 4°C. and formed an intense coral-pink pigment. At higher temperatures pigment production became weaker until almost colorless colonies appeared at 40°C. The color of the S- and R-pink-yellow forms also varied with the temperature. At growth below 25°C. a pink pigment predominated, at 37° or over the colonies were buff colored.

Smooth-white, smooth-brown, and translucent colonies grew at 42°C. but only the S-brown grew at 44°C. The S-brown colonies were colorless at temperatures over 40°C.

Effect of pH. Agar of different pH was prepared by addition of NaOH or HCL. The final pH was determined with the glass electrode before pouring the plates. Sets of plates were seeded with the variants and incubated for four days at 25°. The results are shown in table 1. Due to uncertainties in holding the pH of agar constant over several days, the results of repeated tests

TABLE 1

Differences in ability of the variant forms of a strain of M. tetragenus to grow under adverse conditions in regard to temperature, hydrogen-ion concentration, and on salt-agar

	M-YELLOW	S-YELLOW	M-WHITE	S-WHITE	M-PINK	S-PINK	R-PINK	M-PINK-YELLOW	S-PINK YELLOW	R-PINK-YELLOW	M-BROWN	S-BROWN	R-BROWN	TRANS-LUCENT	BACIL-LARY
44°C.	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
42°C.	−	−	+	+++	−	−	−	−	−	−	−	++	−	+	+
40°C.	++	++	+++	+++	+	+	+	++	+	+	+	++++	+	++	++
37°C.	++++	++++	+++	++++	++++	+++	+++	++++	++++	++++	++++	++++	++++	++++	++++
25°C.	+++	++++	++++	+++	+++	++++	++++	++++	++++	++++	++++	+++	++++	++++	++++
10°C.	+	++	−	−	−	+++	+++	+	+++	++	++	+	+	−	+
4°C.	−	−	−	−	−	++	+	−	+	+	−	−	−	−	−
pH 10.0	+	+	−	−	+	+++	++	++	+++	+	++	−	++	−	+
9 5	++	++	−	−	++	+++	++	++	+++	++	+++	−	+++	−	++
9.0	++	++	−	−	++	++++	++++	++	+++	+	+++	−	++++	−	++
8 5	++++	++++	+	+	+++	++++	++++	++	+++	+++	++++	++	++++	+	+++
7.5	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
6.5	++	++	++++	++++	++++	+	+	++++	+++	+++	++++	++++	++++	++++	++++
6.0	+	+	++	++	+++	−	−	+	+	+	++	++++	+++	+++	++
5.5	−	−	+	+	+	−	−	−	−	−	++	++	++	+	+
5 0	−	−	−	+	−	−	−	−	−	−	+	++	++	+	−
NaCl 10 per cent	−	−		+	−	−						+		+	−
5 per cent	+	+		+	+	+						+		+	+
2 per cent	++	++		++	++	++						++		++	++

++++ = usual growth at pH of 7.5 ± at 25° to 37°C.
+++ = growth slower and less profuse.
++ = scattered colonies.
+ = 1 to 10 colonies.
− = no growth.

varied somewhat. The data in the table represent the average of numerous trials. Here again, with individual exceptions, group differences similar to those noted in the preceding experiment are evident. The yellow, pink and pink-yellow types behaved similarly and grew better toward the alkaline extreme of the range of pH tested. Growth occurred at a pH as high as 10. The white and brown types and the translucent form on the other hand, were able to grow on agar at a pH as low as 5, but failed to grow toward the alkaline extreme. The mucoid-brown and rough-brown forms were exceptional and grew well throughout the whole range tested.

Effect of other environmental conditions. Studies made on the effects of high NaCl concentration, bacteriostatic dyes and O_2 and CO_2 tension were made in the early months of observation before all of the variant forms eventually studied were isolated. Certain conditions made it impossible to repeat the tests with the complete set of variants.

Agar plates containing 2, 5, and 10 per cent NaCl were inoculated with 10 of the variants and grown at 25°C. Growth of all forms on the 2 per cent NaCl was generally poorer than on the control plates. Growth on the 5 per cent plates was scanty, and only a sparse growth of the smooth-white, smooth-brown and translucent forms appeared after 10 days on the 10-per cent plates. The white, brown and translucent forms again showed similarities in behavior as noted before. See table 1.

Agar plates containing acid fuchsin at 1:80 prevented growth of the smooth-yellow and smooth-pink forms. Crystal violet in dilution of 1:200,000 prevented growth of all the forms tested except the mucoid-yellow and mucoid-pink.

Plates inoculated in the usual way with the variant forms were placed in sealed glass cylinders containing approximately 95 per cent oxygen, 95 per cent CO_2, 90 per cent nitrogen and in a Fildes anaerobic jar, all at 25°C. with control plates in room atmosphere.

There were practically no differences in growth or pigment production of the variant forms whether grown in 95 per cent oxygen or in 90 per cent nitrogen as compared with the controls. Carbon dioxide, however, delayed the growth of the S-yellow and

S-pink forms for nine or ten days, but only slightly inhibited the S-white, translucent or S-brown forms. The mucoid forms grew well. When the plates were removed from the CO_2 atmosphere, the S-yellow, S-pink, and S-pink-yellow colonies grew rapidly. On a few occasions the S-white, translucent and S-brown grew better at increased CO_2 tension than in room air. Growth of all variants was delayed and poor in the Fildes anaerobic jar. After one week a faint growth was visible on the sections seeded with cocci from the S-yellow, translucent and S-brown variants; the colonies of the S-white and S-pink forms were microscopic in size; S-brown colonies grew best.

Effect of growth under adverse conditions for long periods. Tests were then made to determine whether or not growth under conditions known to be unfavorable to the optimal growth of a given variant enforced or provoked dissociation into a form better suited to growth in the different environment. For example, the S-yellow and S-pink forms which grew best at lower temperatures were cultivated for 50 days at an average temperature of 41°C. to determine whether transformation to the white, brown or translucent forms would occur. Transfers of the S-yellow forms were made at three or four-day intervals and grown continuously at 41°C. It was necessary to initiate growth of the pink form for 12 to 18 hours at 25°C. at each transfer; scanty growth then continued for several days at 41°C. Heavy inoculation was necessary before growth occurred. Under these circumstances no evidence of dissociation occurred among the S-yellow colonies during the experiment. On a transfer made from the pink culture on the 45th day, one white colony composed of tetrads appeared among the pink forms.

The S-yellow and S-pink forms which grew best on slightly alkaline medium were then transferred 14 times on acid agar adjusted to a pH of 5.4, during a period of 50 days at 25°C. Growth of the S-pink form was slow, scanty and pale; the S-yellow form also grew slowly. No variation was noted among the yellow colonies, but after the twelfth transfer a few S-white colonies composed of cocci appeared among the pink ones. The occurrence of the white colonies, however, was not unusual since they ap-

peared from time to time on favorable media (Reimann, 1936). Growth of the S-yellow and S-pink forms in acid-broth likewise failed to provoke variation. A point colony of the S-yellow form at 41°C. for 25 days developed into a collection of separate yellow and white colonies. The S-pink failed to grow at this temperature. Point colonies of the S-yellow and S-pink forms on agar at pH 5.4 grew slowly and measured 8 mm. after three weeks at 25°C. No sectors of other variant forms appeared.

The S-white, S-brown, and translucent variants were similarly treated; that is, these forms which were found to grow best on slightly acid media were transferred at three to four-day intervals for 50 days at 25°C. on alkaline agar media adjusted to a pH of 8.3 and 9.5. During this time the white form remained unaltered. On a plate made at the twelfth transfer, a few yellow colonies appeared among the white but in no greater numbers than would be expected occasionally on favorable media. The translucent form on successive transfer gradually became more opaque until the whole culture reverted to the white form, as noted previously even under favorable conditions (Reimann, 1936). The S-brown form remained unchanged throughout the experiment. Point colonies of the S-white, S-brown and translucent forms, grew on plain agar at 41°C. After 25 days the white measured 20 mm. in diameter, the translucent 8 mm., and the brown 22 mm.; the latter was not pigmented. Point colonies of the translucent and brown form on agar at pH 8.3 grew poorly; the latter measured 3 mm. after 21 days at 25°C. No unusual sectors denoting variation were noted under these conditions.

The appearance of occasional variant forms when cultures were aged in full strength or diluted plain broth, was described in study IV (Reimann, 1937).

RESISTANCE TESTS

In the following experiments the variant forms which were at hand at the time the experiments were made were subjected to certain procedures regarded as harmful to bacteria and plated on agar plates in the usual manner to determine whether or not they had survived the treatment. The results are shown in table 2.

Resistance to heat and cold. Light suspensions of certain variant forms of approximately similar density in physiological salt solution in separate test tubes were immersed in a water bath at 56°C. After 15, 30, and 60 minutes, plates were seeded from each tube and incubated at 25°C. for ten days. All six forms tested withstood this degree of heat for five minutes. After 15 minutes plates made from the tubes showed a good growth of S-yellow, S-white, S-brown colonies, four S-pink colonies, but no

TABLE 2

Differences in resistance of certain variant forms of M. tetragenus to bactericidal procedures

		SMOOTH-YELLOW	SMOOTH-WHITE	SMOOTH-PINK	SMOOTH-BROWN	TRANS-LUCENT	BACILLI
Heat at 56°C	30 minutes	+	+	−	+	−	+
	60 minutes	+	−	−	−	−	−
Frozen. . .	23 ×	+++	−	+++	+	+	
	45 ×	+++	−	+++	−	−	
Formalin 0.4 per cent	8 minutes	+++	+	+	+	+	+
	15 minutes	+++	+	−	−	−	+
Ultraviolet light	¼ minute	+++	+	+++	+	−	++
	½ minute	++	+	+	+	−	+
	1 minute	+	−	+	−	−	+

++++ = growth of control colonies.
+++ = growth slower and less profuse.
++ = Scattered colonies.
\+ = 1 to 10 colonies.
− = no growth.

translucent forms. After 30 minutes a few S-yellow, S-white, and S-brown colonies grew. Only the S-yellow ones grew after 60 minutes at 56°C.

Heavy suspensions of the six forms in physiological salt solution in test tubes were repeatedly frozen and thawed by placing them on the outside windowsill in temperature ranging between −26°C. and −31°C. until frozen, then thawing rapidly in running warm water. The process was repeated 45 times. All forms survived 22 freezings but after this, first the S-white, then the

S-brown, and finally the translucent forms succumbed. Cocci from the S-yellow and S-pink forms were still viable when the experiment was discontinued.

All six forms survived drying for five weeks in a pistol desiccator over P_2O_5.

Resistance to formalin. Organisms were added to separate tubes of physiological saline solution containing 0.4 per cent formalin. Plates were made after 3, 8, and 15 minutes and incubated at 25°C. All six forms tested survived 3 and 8 minute exposure, but the number of colonies was greatly reduced and growth poor as compared with controls. The S-yellow was least harmed. After 15 minutes, the S-yellow still grew luxuriantly, but only a few S-white and bacillary colonies grew.

Resistance to ultraviolet light. Four sets of plates were seeded in the usual manner but were uncovered and placed so that the agar surface was 12 inches from a mercury quartz arc (Cooper-Hewitt type, 65 watts, 4 amperes). The plates were covered and removed after $\frac{1}{4}$, $\frac{1}{2}$, 1, and 2 minutes exposure, sealed with tape and observed at 25°C. for ten days. After $\frac{1}{4}$ minute exposure good growth of the S-yellow and S-pink occurred, only a few S-white and S-brown colonies appeared and the translucent form failed to grow. Similar results occurred after $\frac{1}{2}$ minute exposure, except that fewer yellow and pink colonies grew. The yellow and bacillary forms alone survived two minutes of exposure. Similar results were obtained after several other trials.

Effect of residence in agar foci in mice. As described in a previous paper (Reimann, 1936), it was evident that growth conditions under these circumstances were unfavorable. All 15 forms disappeared after two or three weeks when embedded in agar and injected subcutaneously in mice or rabbits. Variation occasionally occurred especially among the S-white and translucent forms.

DISCUSSION

The results in general support the theory that bacterial variation enables a given strain of bacteria to maintain growth and life under rather widely different conditions. However, as has been shown, type transformation seems to permit a considerably greater range of growth possibility of a strain than does culture-

phase variation. With certain exceptions, the M, S, and R phases of a given type are similarly limited in ability to grow under unfavorable conditions. The component types of the strain of *M. tetragenus* studied fell into two general groups as regards growth possibilities. One group was composed of the abundant-pigment-producing yellow, pink and pink-yellow types which grew better at lower temperatures and on alkaline media, and the other was composed of the white, brown, and translucent types which grew better at higher temperatures, on acid media, on salt media, and in carbon dioxide. Similar group differences were noted in regard to resistance against certain bactericidal procedures. The yellow and pink types were more resistant to heating, freezing, chemicals and ultraviolet light than the white, brown and translucent types.

While the differences in growth capabilities and resistance of the various forms under varying conditions appear to be slight, yet certain environmental differences of similar magnitude occur in the body during infection and fever which may be of great practical significance in infectious disease and in the field of epidemiology. As yet, however, bacterial variation has not been proved to play a rôle in the cause of or in the recovery from infectious disease. Interesting in this regard is the evidence that the optimal pH, temperature range and CO_2 requirements of the smooth-white form originally isolated from a patient (Reimann, 1935), and the mucoid-white forms obtained from Professor Kisskalt which were weakly virulent for mice, seem to endow the white type with ability to exist *in vivo*. Other pertinent observations have recently been made concerning the pneumococcus type II (Enders and Shaffer, Rich and McKee, 1936). The heavily pigmented types, according to the results of the tests, seem unfitted for growth *in vivo*, and may perhaps be regarded as the so-called "wild" types. The latter types were, however, more resistant to bactericidal treatment. No orderly relationship was noted in regard to certain biological properties of the variant forms such as sugar fermentation or gelatin liquefaction with differences in growth or resistance.

It appears from these studies that the ability exhibited by the numerous variant forms of the strain of *M. tetragenus* in question,

to resist a range of different adverse conditions is a phenomenon which permits adaptation of the strain to many adverse environmental circumstances. Obviously the conditions under which the study was made in test tubes and on agar plates in the laboratory are widely different from the conditions met with in nature, but it would seem that the underlying principles might be similar. The results of the tests however, do not indicate that microbic variation represents a purposeful or orderly attempt on the part of the bacteria to develop variant forms better fitted for growth under new and different circumstances. A few instances, such as the appearance of white colonies among the pink when the latter were grown on acid agar, did favor this theory, but similar white colonies were repeatedly noted even under apparently favorable conditions. The white colonies increased in proportion as transfers on acid agar proceeded, but never outnumbered the pink forms during the period of observation. Furthermore, the yellow form when grown at 41°C. did not dissociate into the white or brown forms which are better suited to growth at this temperature; the white, translucent or brown forms did not change into the yellow or pink when grown on alkaline media, nor did any of the variants when injected into the animal body regularly dissociate into the mucoid-white or smooth-white forms which seem best fitted for existence *in vivo.* The latter observations were similar to those previously noted in studies on the pneumococcus (Reimann, 1927). It would seem then that for reasons unknown, a given bacterium may from time to time give rise to variants. The presence by chance of a certain suitable variant, at a time when the environment is unfavorable to the "original" form, would therefore provide the means for continuing the existence of the strain under the new conditions. From this viewpoint, a variant form especially equipped to exist *in vivo* might become invasive and could be regarded as "virulent" provided circumstances in the host were suitable.

SUMMARY

Sixteen variant forms of a strain of *Micrococcus tetragenus* originally isolated from a patient were classified into five specific

types with their respective culture-phases and two unclassifiable forms. They were grown under a variety of adverse conditions to determine whether or not evidence could be obtained to support the view that microbic variation is a purposeful attempt on the part of the bacterium to adjust itself to a new and changed environment. While the various types seemed to fall into two groups in regard to ability to grow at high temperatures on the one hand, or low temperatures on the other, or in acid or alkaline media and in other respects, no evidence was obtained to show that variation was induced in the direction proper to continue existence of the strain. Variants seemed to appear more or less by chance. It was assumed therefore that if by chance a variant suited to a new and changed environment were present at the proper time, life would be continued by this form. To this extent then microbic variation, especially type transformation, seems to be a phenomenon which permits the continued existence of the strain of *Micrococcus tetragenus* under a wide range of environmental conditions.

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GROWTH INHIBITION OF ESCHERICHIA COLI

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Received for publication December 3, 1936

Growth of bacteria in a medium produces changes which, in many cases, are not favorable to secondary growth in the medium. Reinoculations, even after the original cells have been removed, give rise to relatively poor growth. This inhibitory action, first demonstrated by Garré (1887), has been subsequently confirmed by ample experimental evidence. There is, however, some disagreement as to the cause and nature of the inhibition.

In 1933 Asheshov (1933) described an apparatus which seemed to afford a convenient method for studying the effects of bacterial growth in a fluid medium. The apparatus consisted of two tubes forming two compartments separated by a collodion membrane. If the broth of only one tube were inoculated, any dialyzable substances produced during growth would be found also in the second sterile compartment. This sterile medium, which for convenience will be called "treated medium," could then be examined for inhibiting or stimulating substances.

The apparatus used in the experiments to be reported was similar to that described by Asheshov and consisted of two tubes, as shown in figure 1, each of 170 ml. capacity, separated by a collodion membrane of approximately 30 sq. cm. area. Collodion membranes were prepared by a method similar to Asheshov's, using formic acid in the collodion to increase membrane permeability. Permeability was estimated by measuring the filtering time in a Zsigmondy funnel, and is expressed as the number of seconds required to filter 1 ml. of water per square centimeter of membrane surface under a pressure of 400 mm. of mercury. Membranes with a filtering time of 150 to 200 seconds were used as these were relatively porous yet would retain bacteria. The

twin tubes were assembled and sterilized at 115°C. for 20 minutes with the membranes in contact with fluid, since under these conditions permeability was only slightly affected.

The tubes were set up with membranes, filled with medium, autoclaved and tested for sterility. A heart-infusion broth with 1 per cent Bacto peptone at pH 7.2 was used in all experiments.

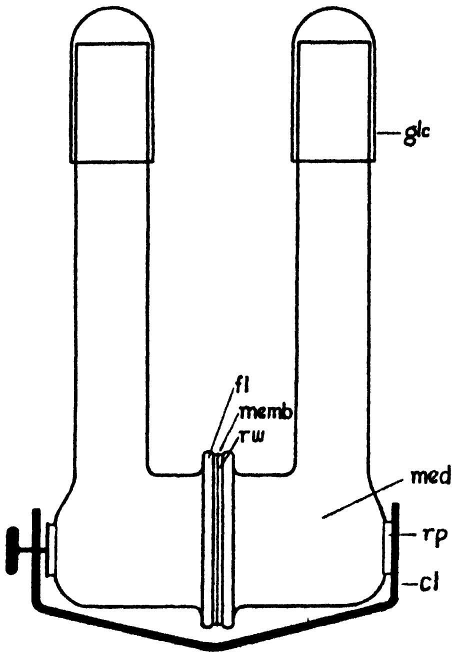

FIG. 1. TWIN TUBES

cl, metal clamp holding tubes together; *glc*, glass cap; *fl*, flange of tube; *med*, media compartment; *memb*. collodion membrane; *rp*, rubber pad; *rw*, rubber washer.

One tube of a pair was inoculated with *Escherichia coli* and after 48 or 96 hours incubation at 37°C. the sterile medium in the second compartment was tested for growth-inhibiting action. This treated medium was readjusted to pH 7.2 and inoculated with a 48-hour culture of *E. coli*. The growth curve was followed by plate counts at suitable intervals and the pH was measured colorimetrically.

Figure 2 shows the growth of *E. coli* in normal and treated medium, with the times in hours represented as abscissae and the logarithms of the population in cells per milliliter as ordinates. The curves are averages from ten experiments. Curve *1* represents growth in normal medium, while *2* and *3* are in 48- and 96-hour treated medium respectively. Both treated media exhibited inhibitory action when compared with the control, although inhibition was apparent somewhat earlier and was stronger in the 96-hour treated broth. In neither case was inhibition strong during the earlier growth phases, but seemed to consist of a decrease in reproduction rate during the logarithmic phase of growth with a

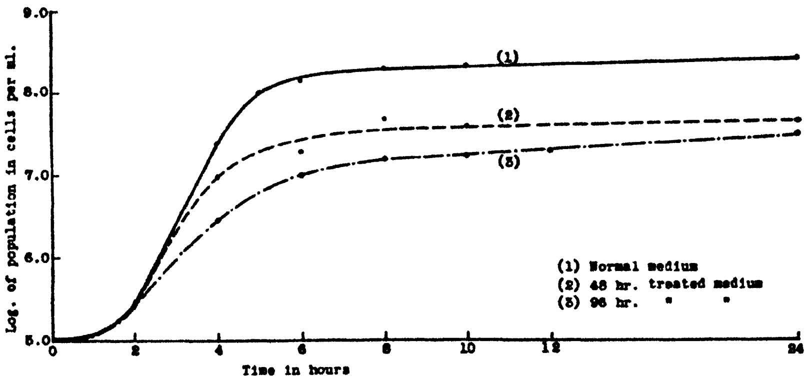

FIG. 2. GROWTH OF E. COLI IN E. COLI TREATED MEDIUM

smaller resulting population. Generation times during the logarithmic growth period calculated on the basis of 100 per cent viability were 21 minutes for normal broth, 30 minutes for 48-hour treated and 46 minutes for 96-hour treated medium. Curves made from normal broth under similar conditions always agreed within one generation and so a difference of more than one generation has been considered a significant difference. Growth in treated medium, as shown in figure 2, was significantly less than the control, beginning at the fourth hour and lasting throughout the experiment which, in some cases, was continued for 50 hours.

Growth in treated medium contained in the twin tubes was exposed to continual dialysis from the originally inoculated tube and

materials could constantly pass between the tubes. To determine if this dialysis produced any significant effect growth curves were followed from broth contained in Erlenmeyer flasks. A culture of *E. coli* was added to 150 ml. quantities of media in 750 ml. flasks. The size of inoculum in these and all subsequent experiments was such as to give the same concentration of organisms, 100,000 to 140,000 per milliliter, as in the tube experiments. Several curves showed the only important change to be a less rapid but longer period of logarithmic growth increase. Final concentrations of bacteria in tubes and flasks were approximately equal. Populations in normal broth at 24 hours as shown by averages from 7 experiments were 2.55×10^8 cells per milliliter for tube cultures and 3.10×10^8 cells per milliliter for flask cultures. Generation times during the logarithmic growth phase were 28 minutes for the flask and 21 minutes for the tube cultures. Since both normal and treated medium in flasks exhibited the longer and slower growth phase, the change could not be attributed to continued dialysis but might possibly have been due to the difference in shape of the container as it affected aeration of the culture.

Curve *1* of figure 3, which is an average of two experiments, represents the population differences per milliliter between normal and 96-hour treated, flask-contained medium. The differences expressed as generations are plotted as ordinates and the times as abscissae. During the four hours following inoculation, treated and normal broth produced nearly equivalent numbers of bacteria. Between the fourth and sixth hours, however, the growth rate in a treated medium decreased so that the sixth hour count was less than 25 per cent of that in normal broth, or about two generations less than in normal broth. This difference of two generations, or more, persisted to the end of the experiment. Generation times for the logarithmic growth period were 37 minutes for treated broth and 28 minutes for control cultures. As figure 3 shows, the first significant population difference occurred at the sixth hour count. This was about two hours later than for the experiments in which twin tubes were used.

The thermostability of the inhibiting substance was tested by

heating 96-hour treated medium in the autoclave at 100° or 125°C. for 15 minutes. A 750 ml. Erlenmeyer flask containing 150 ml. of sterile, treated medium was heated and adjusted to pH 7.2. Normal broth similarly heated served as a control. The results are shown in curve *2* of figure 3. During the first four hours treated broth which had been heated produced a greater population than the control, the fourth hour count being more than twice that of the control or more than one generation greater. By the sixth hour, however, as in the case of curve *1*, the growth rate had so decreased that the populations in treated

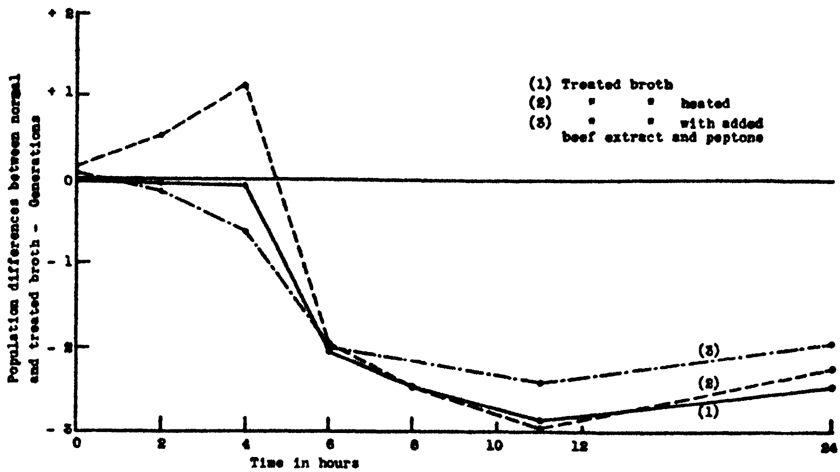

FIG. 3. INHIBITION OF E. COLI IN E. COLI TREATED MEDIUM

broth were two generations less than in the control. All subsequent counts showed a similar population difference.

Curve *3* of figure 3 is an average of two experiments and represents growth inhibition of *E. coli* in treated broth to which beef extract and peptone had been added. The medium was prepared by adding a concentrated solution of beef extract and peptone to 150 ml. of 96-hour treated medium in an Erlenmeyer flask in amounts that gave a final concentration of 1.5 grams of extract and 5.0 grams of peptone per liter. Before inoculation of the "reinforced" medium the reaction was adjusted to pH 7.2. Normal medium, to which was added a similar amount of peptone

and beef extract, served as a control. Curve *3* shows that the population in the reinforced treated medium was significantly less than the control beginning at the sixth hour. Generation times during the logarithmic growth period were 27 minutes in the controls and 34 minutes in the treated broth. Although maximum growth inhibition in a reinforced treated medium (2.3 generations at 11 hours) was less than in a treated medium not containing the additional beef extract and peptone (curve *1*, 2.85 generations at 11 hours), growth in reinforced treated medium was significantly decreased. Furthermore, the differences between curves *1* and *3* were in no case greater than 0.55 generation and are within the experimental error. Growth of *E. coli* in reinforced broth indicates that inhibition occurring in treated medium was not produced by a deficiency of food substances found in beef extract or peptone.

Hydrogen ion concentrations in normal and treated media differed during growth. After inoculation with *E. coli*, the pH of normal broth decreased from 7.2 to a minimum of 6.8 at 8 hours, and then slowly increased to 7.4 at the twenty-fourth hour. After inoculation of treated medium, which had been readjusted to pH 7.2, however, the pH remained constant at 7.2 until the sixth hour and then gradually increased to 7.4. This change in the reaction was quite uniform for the various experiments, even when the medium was contained in flasks, and apparently indicates the absence of some acid-producing material from the treated medium.

A medium exposed to *E. coli* for 96 hours exhibited no specificity of inhibitory action when tested with *Eberthella typhosa* and *Staphylococcus aureus*. Growth of *E. typhosa* in treated broth was significantly lower than in the control, the maximum difference being 2.3 generations. Likewise *S. aureus* was inhibited with a difference of 2.6 generations ten hours after inoculation. The inhibition of *E. typhosa* and *S. aureus* in *E. coli*-treated medium was similar to inhibition of the homologous organism.

These experiments indicate that exposure of a broth medium to the action of *E. coli* by means of the Asheshov apparatus rendered the medium less suitable for growth of some bacterial spe-

cies. Although the differences were not large, growth curves of *E. coli* made in a treated medium which had been heated, apparently showed a slight growth stimulation early in the growth cycle. Later, however, inhibition was apparent and was as great as in unheated broth. The early increased growth rate may possibly have been caused by a destruction of some fraction of the inhibiting substance, or a liberation of nutrient material by heating. Treated broth was not deficient in food substances present in beef extract and peptone, although some acid-producing substance of normal broth was apparently lacking.

The Asheshov apparatus affords a convenient method for exposing a broth medium to bacterial growth while maintaining the medium in a sterile condition, and provides an effective means for the study of some aspects of bacterial metabolism.

SUMMARY

By means of twin tubes, similar to those described by Asheshov, a growth-inhibitory action was demonstrated for *Escherichia coli*. Inhibition could not be attributed to a lack of nutrient materials present in beef extract and peptone, and the inhibitory substance was thermostable and dialyzable. Inhibition was nonspecific since *Eberthella typhosa* and *Staphylococcus aureus* showed decreased growth similar to that of *Escherichia coli*.

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THE ISOLATION OF PROBABLE PATHOGENIC STAPHYLOCOCCI[1]

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Received for publication December 4, 1936

In searching for pathogenic types of staphylococci in cultures taken from suspected pathologic sources, it is customary to plate the cultures on suitable media and select colonies for isolation. Tests of pathogenicity are then applied to the pure cultures. Because of the impracticability of testing all colonies, several typical colonies must be selected in the hope that they will include the pathogenic variants. This method has several disadvantages, among which are the following: (a) colonies of pathogenic variants may not be among those selected; (b) much energy must be expended in isolating and testing non-pathogenic types; (c) some of the cultures selected may be duplicates; (d) it is difficult to estimate the number of colonies of pathogenic staphylococci because they cannot be recognized with certainty in the primary cultures; (e) variations in the size and composition of the bacterial cells make it difficult to secure a uniform test dose; (f) for precise work graded amounts of culture must be injected; (g) the results of animal inoculation tests are frequently confused by effects due to emboli, intercurrent infections, etc.; (h) animal inoculation tests are also influenced by the age and state of nutrition of the animals; (i) there is no sharp demarcation between pathogenic and non-pathogenic staphylococci—making it necessary to establish arbitrary standards; and (j) variations in the method of growing and preparing the culture affect the results of animal inoculation tests. Although the pathogenicity of a strain may vary with different species of animals, it is generally conceded

[1] Aided by grants from the Ophthalmological Foundation, Inc.

that, using the term in its broader sense, a strain which is pathogenic for human beings produces dermonecrotic and lethal effects in rabbits.

The medium to be described was developed to simplify search for pathogenic types. On this medium about 98.5 per cent of strains of the pathogenic type of staphylococcus grew luxuriantly, while about 94 per cent of the non-pathogenic type were inhibited. This medium should be useful for isolation purposes, particularly when a large series of cultures is to be tested.

METHODS USED TO ESTIMATE THE ACCURACY OF THE MEDIUM

In order to determine the reliability of the proposed medium, it was necessary to study the pathogenicity of a large number of strains and test their reactions on the medium. Adequate animal inoculation tests of pathogenicity would have involved a tremendous amount of work. The possibility was considered of substituting *in vitro* tests because hemolysis, coagulase and crystal-violet agar tests of staphylococci had been shown to parallel the lethal effects on rabbits (Chapman, Berens, Peters and Curcio, 1934 and Chapman and Berens, 1935). The positive *in vitro* reactions of strains isolated from pus obtained from sinuses, osteomyelitic lesions, boils, carbuncles, etc. and the correlation of the three *in vitro* properties in a large series of strains indicated that hemolysis, coagulase and crystal-violet agar tests could be applied as *in vitro* indicators of probable pathogenicity. The simultaneous use of several *in vitro* tests, each of which correlated with rabbit inoculation tests, increased the accuracy of interpretation of the results.

CALCULATION OF ERRORS OF DIFFERENT TESTS

In order to make appropriate allowance for possible errors an attempt was made to estimate the degree of accuracy of the *in vitro* tests.

Earlier experiments had demonstrated that animal inoculation tests, particularly when only one animal was used, were not as reliable as is often considered. The possible error was calculated

by injecting graded amounts of killed staphylococcal cultures intravenously into 131 rabbits. If a rabbit died within a few minutes after inoculation, or if it died following the injection of a certain amount of culture while other rabbits injected with larger amounts survived, the deaths were considered as errors. On this basis, 21 of the rabbits (16.3 per cent) gave erroneous results. Therefore, the possibility of such errors must be considered in interpreting the results of animal inoculation tests. Obviously, the results will be influenced by the criteria of pathogenicity. No attempt has been made to study this phase of the problem, it being assumed that, with the reservations just discussed, the animal inoculation experiments mentioned in the previous section are acceptable for comparative purposes.

It was shown (Chapman *et al.*, 1934) that some hemolytic albus strains were not pathogenic while some pathogenic aureus strains were not hemolytic. It also appeared that the coagulase test could be used to complement the hemolysis test. When this combination of tests was applied to the study of 119 strains, the results were parallel with those of rabbit inoculation tests in 81.5 per cent of the strains.

In a later paper (Chapman and Berens, 1935), it was shown that strains could be classified according to the color of their growths on 1:300,000 crystal violet agar. The results of this test agreed with those of rabbit inoculation tests in 27 of 28 strains (96.4 per cent).

In some strains in which the results of *in vitro* and *in vivo* tests did not agree, the discrepancy could be explained on the basis of the 16.3 per cent error of single animal inoculation tests.

PARALLEL BETWEEN HEMOLYSIS, COAGULASE AND CRYSTAL-VIOLET AGAR TESTS

In interpreting the results of *in vitro* tests, aureus strains which produced considerable hemolysis on rabbit blood agar were considered as reacting positively to the hemolysis test. Hemolysis tests were not applied to albus strains. Albus and aureus strains that coagulated oxalated human plasma within 12 hours were considered coagulase-positive. Strains which produced violet or

orange growths on crystal-violet agar in 36 hours were considered positive to the crystal-violet agar test.

The results of the crystal-violet agar test were parallel with hemolytic and coagulating properties in 86.4 per cent of 701 aureus strains and 95.7 per cent of 1012 albus strains (table 1). When the results of the three tests did not agree they could be explained on the basis of an intermediate type of culture or by dissociative

TABLE 1

Comparison of hemolysis and coagulase tests with the crystal-violet agar reaction in 1713 strains of staphylococci

TYPE	STRAINS SHOWING	HEMOLYSIS	COAGULASE	CRYSTAL-VIOLET AGAR	NUMBER	PER CENT
Aureus	Agreement	+	+	+	427	
		0	0	0	28	
		0	+	+	148	
		+	0	+	12	
				Total	615	86.4
	Disagreement	+	0	0	3	
		+	+	0	27	
		0	+	0	19	
		0	0	+	37	
				Total	86	13.6
Albus	Agreement		+	+	91	
			0	0	877	
				Total	968	95.7
	Disagreement		+	0	19	
			0	+	25	
				Total	44	4.3

influences. Even in "pure" cultures of pathogenic strains non-pathogenic variants may appear soon after isolation.

CONSTANCY OF THE IN VITRO REACTIONS

The stability of the reactions was tested by applying the three *in vitro* tests both at the time of isolation and again after storage in the refrigerator for one month. Significant differences were noted in 14 of 200 hemolysis tests (7 per cent), in 12 of 191

crystal-violet agar tests (6.3 per cent) and in 6 of 173 coagulase tests (3.4 per cent).

THE VALUE OF IN VITRO TESTS

When allowances were made for possible errors of the different tests, the agreement with animal inoculation tests and the close agreement between the three *in vitro* tests indicated that they could be used for the study of large numbers of cultures where extensive animal inoculation tests would involve an enormous amount of work.

With due regard to the limitations just discussed, the *in vitro* tests were applied as tests of probable pathogenicity, subject to confirmation by rabbit inoculation tests.

THE DEVELOPMENT OF A MEDIUM FOR THE ISOLATION OF PROBABLE PATHOGENIC STAPHYLOCOCCI

Although the results of crystal-violet agar tests were parallel with those of rabbit inoculation and hemolysis and coagulase tests, it was necessary to inoculate heavily to secure growth on crystal-violet agar. For this reason, it is not suitable for the isolation of staphylococci.

Search for a medium containing a dye which would differentiate pathogenic from non-pathogenic strains, and yet not inhibit pathogenic strains, led to discovery of the value of bromthymol-blue agar. Although the early results with solid media containing bromthymol blue were shown to be due to the indicator property of the dye (Chapman, 1936) it was found that, when the concentration of dye was increased, non-pathogenic staphylococci were inhibited. Best results were obtained with a concentration of 0.17 gram per liter. To determine whether this concentration of bromthymol blue had an inhibitive effect on pathogenic types of staphylococci, swabs from the nose, throat, gum margins, etc. of patients suspected of having chronic diseases were plated on proteose lactose agar containing 0.017 per cent bromthymol blue. Duplicate swabs were plated on rabbit blood agar. The number of colonies on bromthymol-blue agar was estimated and compared with the number of *in vitro* positive colonies isolated from rabbit-blood agar. Parallel results were obtained in 69 of 96 pairs of

swabs and widely different results were obtained in only 3 pairs. Except for a few intermediate size colonies of *in vitro* negative strains, those strains which grew produced colonies as large as those on ordinary media.

TABLE 2

Probable errors of crystal-violet agar and bromthymol-blue agar (pH 6.8) tests

Total number of strains examined 240
Strains showing agreement between bromthymol-blue agar and crystal-violet agar tests 222 (92.5%)

STRAIN NUMBER	TYPE	DISTRIBUTION OF THE 18 STRAINS SHOWING DISAGREEMENT					
		Hemolysis	Coagulase	Crystal-violet agar	Growth on bromthymol-blue agar	Probable error of: Crystal-violet agar test	Probable error of: Bromthymol-blue agar test
3782	Aureus	0	0	+	0	+	
3860	Aureus	+	+	0	+	+	
3892	Aureus	+	+	0	+	+	
3893	Aureus	0	+	0	+	+	
3910	Aureus	0	+	0	+	+	
4067	Albus		+	0	+	+	
4076	Albus		0	+	0	+	
4078	Albus		0	+	0	+	
3737	Aureus	+	+	0	0	+	+
3878	Aureus	0	+	0	0	+	+
3915	Aureus	+	+	0	0	+	+
3917	Aureus	0	+	0	0	+	+
3916	Aureus	+	+	0	0	+	+
3966	Aureus	0	0	+	+	+	+
3961	Albus		+	0	0	+	+
3864	Aureus	+	+	+	0		+
4058	Albus		0	0	+		+
4065	Albus		+	+	0		+
Total errors.						15 (6 4%)	10 (4.3%)

GROWTH OF OTHER BACTERIA ON BROMTHYMOL-BLUE AGAR

In addition to pathogenic staphylococci, other bacteria, particularly members of the colon-aerogenes group, also grew well on bromthymol-blue agar and could not be eliminated by bacteriostatic methods. However, the appearance of colonies of

pathogenic staphylococci is so characteristic that they cannot be confused.

COMPARISON OF ABILITY TO GROW ON BROMTHYMOL-BLUE AGAR WITH THE POWER TO PRODUCE ORANGE OR VIOLET GROWTHS ON CRYSTAL-VIOLET AGAR

In tests of pure cultures, the results of crystal-violet agar and bromthymol-blue agar tests were parallel in 92.5 per cent of 240 strains (table 2). The bromthymol-blue agar test was considered correct in 95.7 per cent of the strains. The majority of errors were due to *in vitro* positive strains that failed to grow, and attempts were made to eliminate this fault.

EFFECT OF CHANGING THE HYDROGEN-ION CONCENTRATION OF BROMTHYMOL-BLUE AGAR

Tests were made to determine whether changes in the hydrogen-ion concentration of bromthymol-blue agar would affect the growth of staphylococci. Various batches of bromthymol-blue agar were prepared and the pH adjusted to 7.0, 8.0, 9.0 and 10.0 before sterilizing. More acid media were too inhibitive for staphylococci. A series of 105 strains was plated on these media and on stock bromthymol-blue agar (pH 6.8 after sterilization). Best results were obtained with the medium which had been adjusted to about pH 10.0 with thymol-blue indicator. The reaction of this medium after sterilization was about pH 8.6. Parallel results were obtained in 98 strains. In 5 instances, *in vitro* positive strains grew better than on stock bromthymol-blue agar. Only one *in vitro* positive and one *in vitro* doubtful strain grew better on the stock medium. It was decided to adjust the medium to about pH 9.6 with the hydrogen electrode before sterilization. After sterilization the reaction was about pH 8.6 with the hydrogen electrode.

COMPARISON OF IN VITRO TESTS OF STAPHYLOCOCCI WITH THE POWER OF THE CULTURES TO GROW ON ALKALINE BROMTHYMOL-BLUE AGAR

A series of 276 strains was plated on this alkaline medium, with the results listed in table 3. The hemolysis test was con-

sidered erroneous in 7.2 per cent of the strains, the crystal-violet agar test in 2.9 per cent, and the coagulase test in 1.1 per cent. The bromthymol-blue agar test gave erroneous results in 7.7 per

TABLE 3

Comparison of different in vitro tests of staphylococci

STRAINS SHOWING	TYPE	HEMOLYSIS	COAGULASE	CRYSTAL-VIOLET AGAR	ALKALINE BROMTHYMOL-BLUE AGAR	NUMBER OF STRAINS	PER CENT OF TOTAL STRAINS
Agreement in all tests	Aureus	+	+	+	+	219	79.3
	Albus		+	+	+		
	Albus and aureus	0	0	0	0		
Probable error of the hemolysis test	Aureus	0	+	+	+	19	7 2
	Aureus	+	0	0	0	1	
Probable error of crystal-violet agar test	Aureus	+	+	0	+	1	2 9
	Aureus	0	0	+	0	3	
	Albus		0	+	0	3	
	Albus		+	0	+	1	
Probable error of bromthymol-blue agar test	Albus and aureus	0	0	0	+	17	6.2
	Aureus	+	+	+	0	1	1.5
	Albus		+	+	0	3	
Probable error of coagulase test	Aureus	+	0	+	+	2	1.1
	Albus		+	0	0	1	
Unclassified	Albus		0	+	+	1	1.8
	Aureus	0	+	+	0	1	
	Aureus	0	0	+	+	3	
Total						276	

cent of the strains. In 17 of the latter instances (6.2 per cent) the error was due to growth of *in vitro* negative strains while, in 4 strains (1.5 per cent), it was due to the failure of *in vitro* positive strains to grow. If the strains growing on bromthymol-blue agar

could be confirmed by other *in vitro* tests, the error of the bromthymol-blue agar method of isolation should be reduced to about 1.5 per cent.

Actual tests of crude cultures indicated that those *in vitro* positive strains that failed to grow in mass culture were not entirely lost because there were a few cells in those cultures which were capable of growing on bromthymol-blue agar.

RESULTS OBTAINED BY PLATING THE ORIGINAL (CRUDE) CULTURES ON BROMTHYMOL-BLUE AGAR AND CONFIRMING THE GROWTHS BY CRYSTAL-VIOLET AGAR AND BY COAGULASE TESTS

Tests were made by plating 81 pairs of swabs from the nose, throat, etc. on bromthymol-blue agar, which had the following composition:

Beef extract	3 grams
Proteose peptone, Difco	5 grams
Lactose	10 grams
Agar	15 grams
Bromthymol blue	0.17 gram
Water to make	1000 cc.

The reaction is adjusted before sterilization to about pH 9.6 with the hydrogen electrode.

In 36 hours, staphylococcal colonies are raised, smooth, entire and opaque. They are similar in morphology to the colonies on ordinary media. The size varies from 1 to 1.5 mm. in diameter depending upon the distribution, colonies on crowded plates being smaller than well-isolated colonies. About 90 per cent are deep yellow while about 10 per cent are gray with a blue tinge. The color does not seem to be significant.

One swab was rubbed first on bromthymol-blue agar and then on rabbit-blood agar. A second swab from the same source was rubbed first on rabbit-blood agar and then on bromthymol-blue agar. This reduced errors due to disproportionate sampling. The inoculum was spread by means of glass spreaders and incubated for 36 hours.

Growths from bromthymol-blue agar were confirmed by (a) transplanting them to crystal-violet agar and incubating for 36

hours and (b) mixing 1 loopful of the growth with 0.5 cc. of oxalated human plasma and incubating overnight. The growths from rabbit-blood agar were transferred to nutrient agar for purification of the different types and *in vitro* tests were applied to the pure cultures. The number of *in vitro* positive colonies was the

TABLE 4

Probable accuracy of different tests of staphylococci

TEST	COMPARISON WITH	NUMBER OF TESTS	PROBABLE ACCURACY
			per cent
Rabbit inoculation	Different amounts of culture	132 rabbits	83.7
Hemolysis	Other *in vitro* tests	276 strains	92.8
	Stability of reaction after storage	200 strains	93 0
Coagulase	Other *in vitro* tests	276 strains	98 9
	Stability of reaction after storage	173 strains	96 6
Hemolysis-coagulase combination	Rabbit inoculation	119 rabbits	81.5
	Crystal-violet agar	1012 albus strains	95.7
		701 aureus strains	86 4
Crystal-violet agar	Rabbit inoculation	28 rabbits	96.4
	Hemolysis-coagulase combination	1012 albus strains	95.7
		701 aureus strains	86 4
	Other *in vitro* tests	240 strains	93.6
	Stability of reaction after storage	191 strains	93.7
Bromthymol-blue agar	Direct isolation (unconfirmed)	96 pairs of swabs	96 9
	Direct isolation (confirmed)	81 pairs of swabs	98.8
	Other *in vitro* tests*	276 strains	92 3

* Most of the error was due to the growth of *in vitro* negative strains. When the growths are confirmed by other *in vitro* tests, the apparent error is reduced to 1.5 per cent, or less.

same by both methods in 71 of the 81 pairs of swabs. In 5 swabs, more *in vitro* positive colonies were recovered by the bromthymol-blue agar method while, in 5 swabs, more *in vitro* positive colonies were recovered by the rabbit blood agar method. Except for one pair of swabs giving an occasional *in vitro* positive colony

on bromthymol-blue agar but a moderate number of *in vitro* positive colonies on rabbit-blood agar the results were essentially similar in both series of swabs.

The deep yellow color carried over from the growths on bromthymol-blue agar sometimes obscured the color of the growths on crystal-violet agar. This was taken into consideration when the results of the crystal-violet agar and coagulase confirmation tests did not agree.

In table 4 an attempt has been made to evaluate the results of different tests. From these results it would appear that, when properly interpreted, hemolysis, coagulase, crystal-violet agar and bromthymol-blue agar tests of staphylococci are sufficiently reliable to be used as indicators of probable pathogenicity.

When the bromthymol-blue agar method of isolation was applied to the study of about 200 crude cultures, 73 of 86 growths on bromthymol-blue agar were considered *in vitro* positive, 7 did not give parallel results to all tests and were considered intermediate in pathogenicity, while 6 growths were considered *in vitro* negative. The proportion of *in vitro* negative strains in this series was similar to that obtained in previous series of tests.

CONCLUSIONS

The power to grow on 0.017 per cent bromthymol-blue lactose agar pH 8.6 is a useful characteristic for the differentiation of staphylococci. It may be used for the isolation of probable pathogenic staphylococci in cases where rabbit inoculation tests are impractical.

The authors wish to express their appreciation to the Difco laboratories for their advice and coöperation in developing the medium and for supplying some of the experimental media.

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FORMATION OF SULFIDE BY SOME SULFUR BACTERIA[1]

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Received for publication December 4, 1936

Previous studies with the strictly autotrophic sulfur bacterium, *Thiobacillus thiooxidans*, growing on elemental sulfur have shown that the sulfur is rapidly oxidized to sulfate without the accumulation of intermediate products (Waksman and Starkey, 1923). However, there is no evidence that the transformation does not take place in several steps (Buchanan and Fulmer, 1930). The possibility has been suggested that the sulfur undergoes some change before entering the bacterial cells, being either reduced to sulfide or oxidized to one of the thionic acids.

The strictly autotrophic sulfur bacterium, *Thiobacillus thioparus*, transforms thiosulfate to sulfate and a material generally considered to be elemental sulfur (Starkey, 1935b). Recently von Deines (1933b) claimed that the sulfur-like substance secreted by the larger sulfur bacteria is not elemental sulfur, but a highly sulfured polysulfide. This substance has long been considered to be the same as that formed by *T. thioparus*. In previous studies with this organism and other bacteria which oxidize thiosulfate, no evidence of sulfide formation was detected (Starkey, 1935b).

In this report consideration is given to the possibility of some transformation of elemental sulfur preceding its passage into the cells of sulfur bacteria and to the possibility that the precipitate formed by *T. thioparus* is a sulfide (Starkey, 1936).

CHARACTERIZATION OF THE SULFUR MATERIAL

In 1870, Cramer first reported on the chemical properties of the globules occurring in cells of some of the large, filamentous,

[1] Journal Series paper of New Jersey Agricultural Experiment Station.

sulfur bacteria. They were insoluble in HCl but soluble in absolute alcohol and in carbon bisulfide; he concluded that the material was sulfur. F. Cohn (1875) observed further that when the cells were heated dry on a glass slide, the globules coalesced to form larger yellow drops giving off sulfurous vapors. Étard and Olivier (1882) found that the globules were soluble in ether and in chloroform. Zopf (1884) characterized the sulfur occurring in filaments of *Beggiatoa* as highly refractive, glistening, round granules, soluble in absolute alcohol, carbon bisulfide, warm potash and warm sodium sulfite, and in nitric acid and potassium chlorate at ordinary temperatures.

Winogradsky (1887) referred to the cells of *Beggiatoa* as follows: "They oxidize H_2S and store up sulfur in the form of small globules which consist of soft amorphous sulfur that never is transformed into the crystalline condition in the living cells" (p. 589).[2] He concluded that these oily-appearing globules are pure sulfur; the globules changed to crystalline sulfur after death of the organism. By heating the cells in water at 70°C., the numerous small globules contained in each cell coalesced to form a single large droplet; in the living cells, plasma membranes keep the globules apart. This was considered evidence that the material was not solid sulfur since the latter melts only above 100°C.

Gasperini (1898) noted that the globules disappeared when treated with acetic acid, and concluded therefore that the material was not pure sulfur. Corsini (1905) discovered that the acetic acid caused the soft oily globules to change to crystals of rhombic sulfur. He confirmed most of the reactions of the sulfur with the previously mentioned reagents and noted further that it dissolved in benzene and in xylol, that it was not affected by strong acids (HCl, H_2SO_4, and HNO_3) in the cold, but was oxidized by hot nitric acid.

When the cells are treated with aceto-carmine, the sulfur crystallizes outside of the cells (Ellis, 1932) the same as during treatment with acetic acid or other materials which cause death of the organism (Winogradsky, 1887). The same author found that watery picric acid also causes the sulfur to crystallize. When

[2] Translated.

treated with a solution of silver nitrate, the sulfur droplets change to Ag_2S (Monti, 1935).

The globules produced by *T. thioparus* from thiosulfate have the same appearance as those produced by the larger sulfur bacteria (Starkey, 1935a). Nathansohn (1902) described the material as droplets of oily amorphous sulfur occurring outside of the small bacterial cells. The globules dissolved in chloroform and in carbon bisulfide and gave off sulfur dioxide when heated. Beijerinck (1904a and b) dissolved the globules in benzene. They do not appear to be affected by strong acids in the cold, but are decomposed by strong alkalis and warm nitric acid. There is no evidence that the globules produced by the larger colorless sulfur bacteria, by the purple bacteria, or by *T. thioparus* differ chemically.

The reactions of the globules with mineral acids, alkalis, acetic acid, and the various solvents were confirmed by von Deines (1933b). His claim that the material is a polysulfide depends principally upon the following observations: by microchemical tests the material was found to be 99 per cent sulfur; by repeated washing it became emulsified, a property which he believed was characteristic of highly sulfured polysulfide and not elemental sulfur; when the sulfur was washed with dilute hydrochloric acid and then held under reduced pressure, it puffed up with bubbles of hydrogen sulfide gas.

EXPERIMENTAL

Medium I was used for cultivation of *T. thioparus* and medium II for *T. thiooxidans*.

	Medium I *grams*	*Medium II* *grams*
Distilled water	1000.0	1000.0
Sulfur		10.0
$Na_2S_2O_3 \cdot 5H_2O$	10.0	
$(NH_4)_2SO_4$	0.1	0.3
K_2HPO_4	2.0	
KH_2PO_4		3.0
$CaCl_2 \cdot 2H_2O$	0.1	0.25
$MgSO_4 \cdot 7H_2O$	0.1	0.5
$FeCl_3 \cdot 6H_2O$	0.02	0.02
$MnSO_4 \cdot 2H_2O$	0 02	
pH	7.8	4.8

For medium I the thiosulfate and ammonium sulfate were sterilized separately; all of the constituents were sterilized under pressure. The flasks of medium II were sterilized in flowing steam for thirty minutes on each of three successive days. Thiosulfate was determined by titration of aliquot portions (from 5 to 25 cc.) with 0.01 normal iodine solution.

Ash content of the precipitate

The following experiment was performed to determine whether or not the material precipitated by *T. thioparus* contains any substance, such as sulfide, which will combine with appreciable amounts of metallic cations.

TABLE 1

Residues from sulfur material precipitated from thiosulfate by T. thioparus

	SOLUTION MADE ALKALINE	SOLUTION MADE ACID
pH before inoculation	7.8	7.8
pH after growth	5.2	5.2
	mgm.	*mgm.*
Initial thiosulfate-sulfur in 900 cc	2,286 3	2,286.3
Thiosulfate-sulfur in 900 cc. after growth	4.8	9.0
Weight of material extracted by CS_2	770.1	772.1
Weight of residue after ignition	0.1	−0.8

The bacterium was grown in medium I in 300-cc. amounts in 1-liter Erlenmeyer flasks. After 28 days, the thiosulfate was practically all decomposed and the reaction had changed from the initial pH 7.8 to 5.2 as indicated in table 1. The solutions in three flasks were mixed together, adjusted to pH 9.0 with NaOH, and kept for one hour before filtration. The precipitate was then collected on a filter paper and washed with 500 cc. of distilled water. The solutions of three other flasks were mixed together and then adjusted to pH 3.0 with acetic acid. After standing for one hour, the solution was filtered and the precipitate washed with 500 cc. of distilled water.

It was assumed that the acid treatment would remove bases

which were associated with any sulfide contained in the precipitate. Upon subsequent ignition there should be no ash. Any sulfide which was present in the solutions which were made alkaline should form compounds which would leave a residue on ignition.

The papers and contents were air-dried and the major portion of the precipitate was transferred to flasks and treated with 100-cc. portions of CS_2 for 24 hours. The solution was filtered, the filtrate evaporated in small crucibles, and the residue dried at 65C°. The material had the appearance of crystals of pure rhombic sulfur. After ignition there was no residue in the case of the material originating either from the acid or alkaline media as shown by table 1. The slight positive or negative differences are within the error of the determination using crucibles weighing about 15 grams each. If any sulfide was contained in the sulfur material, it was not detected by this method. If there had been any appreciable amount of sulfide, it should have been detected in the relatively large amount of material used. Sulfides of the alkalis are not appreciably soluble in CS_2, but the fact remains that, although the sulfur was readily soluble in CS_2, no material leaving an ash residue was dissolved. Since in this experiment it is only the globules which are being characterized, any sulfide which might have been removed during the washing need not be considered.

Influence of the precipitate on the iodine titration of the medium

Medium I, in 100-cc. portions in 250-cc. Erlenmeyer flasks, was inoculated with *T. thioparus*. After 23 days, 20-cc. portions were titrated with standard iodine solution. Similar portions were titrated after filtration to remove the precipitate. As shown in table 2, there was no material reacting with iodine after the thiosulfate had been decomposed; the amount of iodine required to give a blue color to the starch indicator was only one drop for 20 cc. of culture solution.

Twenty cubic centimeters of the uninoculated medium contained 45.6 mgm. of thiosulfate-sulfur. If, after decomposition by the bacterium, two-fifths of this was precipitated sulfur as

determined in previous studies (Starkey, 1935b), there would be 18.2 mgm. of the precipitate in the aliquot used for titration. If it is assumed that only one per cent of this was in the form of sulfide as H_2S, the amount of H_2S would be 0.182 mgm. Since

$$H_2S + I_2 = 2\,HI + S$$

the amount of iodine required for its oxidation would be 1.36 mgm. One cubic centimeter of the iodine solution which was used contained 1.25 mgm. of iodine, consequently 1.09 cc. of the iodine solution would have been required to effect the oxidation. An amount of sulfide reacting with such a quantity of iodine would be detected easily by the method used. It thus appears

TABLE 2

Titration of solutions before and after removal of the precipitate

	CONTROL MEDIUM	INOCULATED MEDIUM
pH	7 8	4 4
	cc.	*cc.*
Titration of 20 cc. with iodine solution before filtration*	72.20 72 15	0 05 0 05
Titration of 20 cc. with iodine solution after filtration*	72.25 72 25	0.05 0 05

* Iodine solution was 0.00986 normal.

clear that, at least in old cultures, the material contains no appreciable amount of sulfide.

To further test for reducing properties of the precipitate, solutions supporting growth of *T. thioparus* were used at periods preceding complete disappearance of thiosulfate. Medium I was used in 125-cc. amounts in 1-liter Erlenmeyer flasks. Periodically, untreated and filtered portions of one control and one inoculated solution were titrated with standard iodine solution. Since oxidation in the culture continues during the period of analysis, 1 cc. of toluol was added to each flask of medium before starting the analysis. Toluol does not affect the titration. In making the analyses, the 20- or 25-cc. aliquots received 5 cc. of 10-per-cent acetic acid and an excess of 0.01-normal iodine solution, the

excess being titrated with standard thiosulfate solution. In all cases precipitate appeared in the inoculated solutions, the amount increasing with the oxidation of the thiosulfate. Any sulfide contained in the precipitate should react with the iodine to show differences in the titrations of the solutions before and after filtration. The data in table 3 give no indication that the reaction with iodine was at all affected by the precipitate. Any sulfide formed during growth of the organism was therefore present in very small amounts and must be of little importance in characterizing the precipitate.

TABLE 3

*Amounts of iodine solution used to oxidize equal amounts of unfiltered and filtered culture solutions**

TREATMENT	INCUBATION	UNINOCULATED	INOCULATED
	days	*cc.*	*cc.*
Unfiltered†	5	80 30, 80 35	67.90, 67.80
Filtered†	5	80 20, 80 20	67 85
Unfiltered‡	12	102 00, 102 25	4 15, 4 15
Filtered‡	12	102 00, 102.20	4 15, 4 15
Unfiltered‡	21	103 30, 103 30	5 55, 5 50
Filtered‡	21	103 60, 103 60	5 55, 5 55

* Iodine solution was 0.00986 normal. Duplicate determinations on each solution.

† 20 cc. aliquots.

‡ 25 cc. aliquots.

In further testing for the presence of sulfide, the method of Kurtenacker and Bittner (1924) was used. This involves three procedures: (*a*) One part of solution (in this case 25 cc.) was added to an excess of standard iodine solution, acidulated with acetic acid, and the residual iodine titrated with standard thiosulfate. The iodine reacts with all sulfide, sulfite, and thiosulfate present. (*b*) A second portion (25 cc.) was placed in a 250-cc. glass-stoppered bottle with 15 cc. of 10-per-cent zinc acetate solution and diluted to about 150 cc. Five cubic centimeters of formaldehyde were added to bind any sulfite present, and the solution was acidulated with acetic acid. An excess of stand-

ard iodine solution was added, the solution shaken, and the excess iodine titrated with standard thiosulfate. The iodine reacts with all sulfide and thiosulfate. (*c*) A third portion of the solution was placed in a 100-cc. volumetric flask with 15 cc. of the zinc acetate solution and made up to volume. After thoroughly mixing, the contents were filtered. An aliquot was treated with

TABLE 4

*Amounts of iodine solution required to oxidize unfiltered and filtered solutions in testing for sulfide, sulfite and thiosulfate**

TREATMENT AND SUBSTANCES DETERMINED†	INCUBATION	UNINOCULATED	INOCULATED
	days	*cc.*	*cc.*
Unfiltered for S_2O_3, S, SO_3	6	100 35	78.65
Unfiltered for S_2O_3, S	6	99 90	78 55
Unfiltered for S_2O_3	6	100 30	78 60
Filtered for S_2O_3, S, SO_3	6	100 45	78 65
Filtered for S_2O_3, S	6	99 95	78 50
Filtered for S_2O_3	6	100 40	78.80
Unfiltered for S_2O_3, S, SO_3	13	102 00	3 40
Unfiltered for S_2O_3, S	13	101 50	3.40
Unfiltered for S_2O_3	13	102 00	3.40
Filtered for S_2O_3, S, SO_3	13	102 10	3.40
Filtered for S_2O_3, S	13	101 60	3 35
Filtered for S_2O_3	13	102 00	3.40
Unfiltered for S_2O_3, S, SO_3	19	103 10	0 15
Unfiltered for S_2O_3, S	19	102 65	0.10
Unfiltered for S_2O_3	19	103 05	0 20
Filtered for S_2O_3, S, SO_3	19	103.15	0 10
Filtered for S_2O_3, S	19	102 65	0 10
Filtered for S_2O_3	19	102 95	0 20

* Iodine solution was 0.00986 normal.

† Thiosulfate—S_2O_3; sulfide—S; sulfite—SO_3.

5 cc. of formaldehyde, acidulated with acetic acid, and titrated with standard iodine solution. This determination indicates the amount of thiosulfate. The difference between (*a*) and (*b*) indicates the sulfite content; the difference between (*b*) and (*c*) indicates the sulfide content. In the present case, lack of differences between the three determinations indicates that only thiosulfate was present, and that there was no sulfide or sulfite.

The results in table 4 are reported as amounts of the standard iodine solution used in the titration of equal amounts of culture solutions; corrections have been applied for the amounts of standard thiosulfate used. The data clearly indicate that there are no significant differences in the determinations at any one period, whether the solutions are unfiltered or filtered to remove the precipitate before titration. Neither do the results show any detectable amount of sulfide or sulfite at any of the three periods which represent: (*a*) an early stage of bacterial growth, (*b*) a period when the thiosulfate is nearly all decomposed, and (*c*) a period following complete decomposition of the thiosulfate. If any sulfide was formed in the cultures it failed to accumulate.

Evolution of sulfide by T. thioparus

Although the previous experiments did not indicate that there was any sulfide in solutions supporting growth of *T. thioparus*, it is conceivable that very small amounts of sulfide were produced. Therefore more sensitive tests were sought.

The nitroprusside test was found to give a strong, although quickly fading, violet color in 5 cc. of a solution containing 0.05 mgm. of sulfide-sulfur, or 1 part in 100,000. A light violet test which persisted but a few seconds was detected in 5 cc. of a solution containing slightly more than 1 part in 500,000. This was the limit of effective use of the test. The iodine titration as used would reveal 1 part of sulfide-sulfur in 100,000 with 5-cc. samples, but this was close to the limit of its usefulness.

In making the nitroprusside test, 5 cc. of the solution was made distinctly alkaline with ammonium hydroxide, 0.5 cc. of 5-percent sodium nitroprusside was added, and the color quickly noted. Modifications suggested by Walker (1925) and Giroud and Bulliard (1933) were also tried with similar results.

Lead acetate paper, a very sensitive indicator for sulfide, was used to test for evolution of sulfide from the culture media during growth of the bacterium. A strip of freshly prepared moist paper was held in place by the cotton plug so that the lower end was about 5 mm. from the surface of the solution medium.

In another test, a 10-cc. portion of the culture solution was

boiled for 3 minutes with a lead acetate paper hanging to within 2 cm. of the surface of the gently boiling solution.

Table 5 presents some of the results from cultures of *T. thioparus*. Similar determinations on uninoculated solutions were made regularly. In none of the solutions, either sterile or inoculated, was there any color reaction with nitroprusside. A faint darkening of the lower edge of the lead acetate papers over

TABLE 5

Sulfide production by T. thioparus growing in thiosulfate solutions

INCUBATION	THIOSULFATE DECOMPOSED IN INOCULATED SOLUTIONS	REACTION (pH) OF INOCULATED SOLUTIONS*	NITROPRUSSIDE TEST		BLACKENING OF Pb-ACETATE PAPERS DURING INCUBATION		BLACKENING OF Pb-ACETATE PAPERS BY HEATED SOLUTIONS	
			Uninoculated	Inoculated	Uninoculated	Inoculated	Uninoculated	Inoculated
days	*per cent*							
2	3.5	8 0	−	−	−	−	−	−
3†	6.6	8.0	−	−	−	−	−	+
4	15 6	7 8	−	−	−	±	−	+
5	18.8	7 3	−	−	−	±	−	++
6	23 9	7 3	−	−	−	±	−	++
7	30 8	7 2		−		+		++
9	41 3	6 9	−	−	−	+	−	++
10	66 3	6 8		−		++		++
11	79.7	6 6	−	−	−	++	−	++
12	98 1	6 2		−		++		++
13	100 0	5 0	−	−	−	++	−	+
15	100 0	4 8	−	−	−	++	−	+
16	100 0	4 8		−		++		+
18	100 0	4 8	−	−	−	++	−	−
20	100 0	4.8	−	−	−	++	−	−
23	100.0	4 8	−	−	−	++	−	−

* pH of uninoculated medium was 8.0.

† Definite evidence of precipitation of sulfur in inoculated solutions at this period; the amounts increased with growth at later periods.

the inoculated solutions appeared on the fourth day. The color became darker and gradually spread some distance (4 to 8 cm.) up from the lower end. It was very apparent that sulfide was formed during growth. Sulfide was evolved in small amounts even while the medium was alkaline, and in somewhat greater amounts after the reaction became slightly acid; there was scarcely any change after the reaction had dropped to pH 6.6.

A fresh test paper was substituted for the darkened one in the flask of a culture which had been incubated for 17 days. At this time all of the thiosulfate had been decomposed. No darkening of the new test paper appeared even after 20 more days, suggesting that the active bacterial cells produced the sulfide.

In no case was there even slight darkening of the papers in the sterile flasks. Sulfur precipitation invariably accompanied growth of the organism on thiosulfate and there was an abundance of precipitate before a pronounced sulfide reaction was observed. It is likely that the sulfide originated from this sulfur.

The samples from the uninoculated solutions liberated no sulfide upon being heated. With similar treatment, the unfiltered inoculated solutions gave a slight test for sulfide after incubation for 3 to 4 days; a strong test was obtained after incubation for from 5 to 12 days. Later the test was weaker or disappeared. However, it seems unlikely that this test on the heated solutions indicates that sulfide was present in the culture media; the following evidence suggests that the sulfide was formed from the sulfur contained in the medium during the heating process. The following tests refer to solutions heated to detect sulfide.

One of the culture solutions which gave a strong sulfide test failed to give any blackening of the acetate paper after removal of the precipitate by filtration. Culture solution oxidized by iodine still gave a test for sulfide. A culture solution of pH 5.0, in which all thiosulfate had been decomposed, gave no test for sulfide; the same solution adjusted to pH 7.0 gave a positive test for sulfide; the test was still stronger after the solution was adjusted to pH 8.0. Neither uninoculated medium nor culture solution in which the thiosulfate had undergone decomposition gave a test for sulfide, but a mixture of the two gave a positive test. Uninoculated thiosulfate medium receiving a small amount of either rhombic or precipitated sulfur liberated sulfide upon being heated. Rhombic sulfur reacted the same as the sulfur precipitated by the bacteria in that there was little or no test for sulfide in a buffered solution at pH 5.4, a positive test at pH 7.0, and a strongly positive test at pH 8.0. These results show that the sulfide evolved from culture solutions which were being

heated originated from the sulfur through reaction with the solution, the reaction being favored by alkaline solutions, but still taking place in weakly acid solutions.

Formation of sulfide by T. thiooxidans.

Thiobacillus thiooxidans, the sulfur bacterium growing under very acid conditions (Waksman and Starkey, 1923, Starkey,

TABLE 6

Sulfide production by T. thiooxidans growing on elemental sulfur

INCUBATION	NaOH (0.07 N) USED FOR 5 CC. OF CULTURE*	NITROPRUSSIDE TEST		BLACKENING OF Pb-ACETATE PAPERS DURING INCUBATION		BLACKENING OF Pb-ACETATE PAPERS BY HEATED SOLUTIONS†	
		Uninoculated	Inoculated	Uninoculated	Inoculated	Uninoculated	Inoculated
days	*cc.*						
4	4 8	–	–	–	–	±	±
5	5 0	–	–	–	–	±	±
6	6 9		–		–		±
7	6 6	–	–	–	–	±	±
8	8 8		–		–		±
10	9.1	–	–	–	±	±	±
11	10.1		–		±		±
12	13 8	–	–	–	±	±	±
15	18 6	–	–	–	+	±	±
18	21.6	–	–	–	+	±	±
20	25 2	–	–	–	+	±	±
23	26 6	–	–	–	++	±	±
25	26.7	–	–	–	++	±	±

* Titer of 5 cc. of uninoculated medium was 1.70 cc. NaOH. Reaction of uninoculated medium was pH 4.6. Reaction of inoculated medium was pH 1.6 at 4 days and below pH 1.2 at all later periods.

† Solutions were boiled gently for 5 minutes. The test was weak in all cases, no differences being noted between uninoculated and inoculated media.

1925), was studied for sulfide formation in medium II. Lead acetate papers were suspended above the culture solutions. Sulfide formation was very similar to that with *T. thioparus* (table 6). The acidity increased rapidly, due to the oxidation of the sulfur to sulfuric acid. No indication of sulfide was shown by the nitroprusside test on either the sterile or inoculated solutions. The lead acetate papers began to show slight darkening at the

edges in 10 days; the color became more intense and general as incubation continued, and was quite strong after 23 days. There was no coloration of the papers in the flasks of sterile medium.

The results clearly indicate that sulfide is produced by *T. thiooxidans*, that little is evolved before growth is well advanced, and that no appreciable amount persists in the solution medium. After 25 days, the darkened papers were replaced. Within one day there was slight coloration and this continued to darken, showing a strong test in 5 days. Sulfide apparently is formed continuously during the oxidation of sulfur to sulfuric acid by *T. thiooxidans.*

None of the solutions contained any detectable amount of reducing substances which reacted with iodine; one drop of the 0.01-normal iodine solution invariably gave a deep blue color to the starch indicator in the 5-cc. portions of the culture solutions. The tests for sulfide on the unfiltered heated solutions were very slight and were the same on both sterile and inoculated solutions. The elemental sulfur contained in the medium commonly reacted in the hot solutions to give a weak test for sulfide. No doubt this sulfide was produced during the test, but only in small amounts due to the relatively high acidity of the solutions.

Sulfide formation by other bacteria which decompose thiosulfate

Thiobacillus novellus, a facultative autotroph oxidizing thiosulfate to sulfate (Starkey, 1935b), produced no sulfide while growing on the thiosulfate medium for 20 days. Since no elemental sulfur was produced during this period it is not surprising that sulfide was not formed.

Other bacteria, referred to as cultures B, T, and K in previous communications (Starkey, 1934a and b, 1935a and b), oxidize thiosulfate to tetrathionate which breaks down into various other sulfur compounds by secondary reactions. Cultures of these bacteria gave rise to sulfide in some cases and not in others. Sulfur generally appears among the secondary decomposition products in the medium, and where there was evidence of sulfur precipitate, sulfide was formed. This emphasizes the fact that sulfide is produced by the various bacteria only when elemental sulfur is present in the medium.

Mechanism of sulfide formation

The following experiments were performed to discover the means of sulfide formation in the cultures.

Neither rhombic nor precipitated sulfur gave rise to sulfide in the sterile acid inorganic medium (medium II). In most cases, both rhombic and precipitated sulfur have been used in parallel experiments, since von Deines (1933a) claimed that precipitated sulfur obtained from various chemical reactions is a highly sulfured hydrogen polysulfide. In the present studies no qualitative differences have been noted between the two materials; precipitated sulfur is somewhat more reactive than the rhombic sulfur, probably due to its finer state of division.

In order to determine whether or not sulfide is formed from sulfur in the alkaline thiosulfate medium, the following three media were used in 100-cc. amounts in 250-cc. Erlenmeyer flasks; five flasks were employed in each case: (*a*) medium I, (*b*) medium I with about 0.5 gram of precipitated sulfur per flask, (*c*) medium I with about 0.5 gram of rhombic sulfur per flask. The sulfur was sterilized in flowing steam and added to the sterile solutions. The media were shaken to favor wetting of the sulfur. Since it did not become readily moistened and practically all remained on the surface, a second series was arranged like the first except that a small amount of lecithin (1 cc. of a sterile solution of approximately 0.25-per cent concentration) was added to each flask. A strip of lead acetate paper was suspended over each solution. In none of these sterile solutions was there any positive test for sulfide even after a period of 20 days. One flask which contained rhombic sulfur and lecithin became contaminated with *Aspergillus niger*. In this flask the test paper showed darkening, clearly indicative of sulfide formation. These results indicate that sulfur is not hydrogenated in the sterile culture solutions. However, sulfide was formed in similar solutions not only by the sulfur bacteria already mentioned, but also by *A. niger*.

In order to determine how general hydrogenation of elemental sulfur might be among microörganisms under the conditions of the preceding experiments, five cultures each of common bacteria, actinomycetes, and fungi were used. They were inoculated

into media prepared the same as (*a*)–(*c*) of the preceding experiment. No organic material was added. After inoculation with a small amount of cell material, a strip of test paper was sus-

TABLE 7

*Sulfide production by heterotrophic microörganisms in thiosulfate medium in presence or absence of elemental sulfur**

ORGANISM	PERIOD OF INCUBATION						
	2 days	5 days	6 days	8 days	10 days	14 days	20 days
Thiosulfate medium alone							
Act. californicus	0	0	0	0	0	0	0
Act. flavovirens	0	0	±	±	1	1	1
Act. griseus	0	1	1	1	1	1	2
Act. violaceus-ruber	0	1	1	1	1	1	2
Asp. flavus	0	0	0	±	1	1	1
Humicola sp	0	0	0	0	0	0	0
Rhizopus sp	0	1	2	2	2	2	3
Thiosulfate with rhombic sulfur							
Act. californicus	0	0	0	0	0	0	0
Act. flavovirens	0	0	0	0	0	0	0
Act. griseus	±	1	1	2	2	3	4
Asp. flavus	0	0	0	±	±	±	±
Humicola sp	0	0	0	0	±	±	±
Rhizopus sp	0	1	2	2	2	2	2
Thiosulfate with precipitated sulfur							
Act. californicus	0	0	±	1	1	1	1
Act. flavovirens	1	2	2	2	2	2	2
Act. griseus	1	1	2	2	2	3	4
Act. violaceus-ruber	1	2	2	2	4	4	6
Asp. flavus	0	0	±	1	1	2	2
Humicola sp	0	0	1	1	1	2	2
Rhizopus sp	1	2	2	3	3	4	6

* 0 = no test; ± = very slight; 1, 2, 3, 4, etc., indicate increasing intensity of dark coloration of the test papers.

pended above the solution in each flask. None of the uninoculated media gave a test for sulfide. None of the bacteria, including *Sarcina lutea*, *Bacterium radiobacter*, *Azotobacter chroococcum*,

Cellulomonas fima, and *Bacillus cereus* formed any sulfide. *Actinomyces pheochromogenus*, a species of *Trichoderma*, and a species of *Penicillium*, likewise gave no reaction. Those showing some indication of sulfide production are noted in table 7.

The nitroprusside test was negative on all solutions after incubation for 20 days. There was no indication of appreciable decomposition of thiosulfate on titration with iodine solution, and the pH was the same in the control and inoculated solutions (8.0).

In no case was there appreciable growth of the organisms since no organic substances were added to the medium; some fungal hyphae or small flocs of growth of the actinomycetes were detected by careful inspection. Even with this limited amount of cell material there was pronounced sulfide formation in some cases, particularly in the medium containing precipitated sulfur. The test papers became black in the flasks of this medium inoculated with *Rhizopus* sp. and *Actinomyces violaceus-ruber*. There was a strong test with *Actinomyces flavovirens* and *Actinomyces griseus* and weaker tests with three other orgnisms. The reaction was slower in developing and not so intense from the rhombic sulfur. Even in the absence of elemental sulfur some of the organisms produced sulfide, but in only one case (*Rhizopus* sp.) was there more than a weak test. Apparently some of these organisms are able to reduce thiosulfate to sulfide. Such reduction by bacteria was reported by Hölschewnikoff in 1889, and a short time later was noted by Petri and Maassen (1893) for numerous bacterial species. More recently it has been shown that this reduction can be effected by many bacteria (Beijerinck, 1900 and 1904b; Sasaki and Otsuka, 1912; Lederer, 1913; Tanner, 1917; Tarr, 1933), yeasts, and filamentous fungi (Kossowitz and Loew, 1912; Neuberg and Welde, 1915; Tanner, 1918; Armstrong, 1921; Korsakova, 1933, see also Bunker, 1936). These studies were made almost invariably in organic media supporting active growth of the organisms, providing conditions more favorable for reduction of thiosulfate than those used in the present experiments.

The formation of sulfide from elemental sulfur seems to be conditioned by the presence of appreciable amounts of cell material

in proximity to the sulfur; the tests have been most readily detected when the sulfur and cells are both on the surface. The following experiment was performed in order to establish this relationship more definitely. Five different media were used: (*a*) thiosulfate medium I, (*b*) medium I with rhombic sulfur, (*c*) medium I with precipitated sulfur, (*d*) medium I containing rhombic sulfur but no thiosulfate, (*e*) medium I with precipitated sulfur but no thiosulfate.

The cultures used included not only those which gave positive tests for sulfide in the previous tests, but also those which produced little or no sulfide. Considerable amounts of cell material were used. For the bacteria, the cells from a 4-day old culture on a nutrient agar slant were removed with a platinum blade and suspended in the solution before adding the sulfur. The fungi and actinomycetes were grown for 4 and 5 days respectively in 40-cc. portions of the following modified Krainsky medium in 125-cc. Erlenmeyer flasks: tap water 1000 cc., glucose 10 grams, asparagin 1 gram, K_2HPO_4 0.5 gram, $MgSO_4 \cdot 7H_2O$ 0.5 gram, $FeCl_3 \cdot 6H_2O$ 0.15 gram, $CaCl_2 \cdot 2H_2O$ 0.15 gram. After incubation, the medium was decanted, the cell material washed with two changes of sterile tap water, and transferred to the flasks of the media (*a*)–(*e*), all of the growth in one flask being used for each test. The weight of the cell substance of the fungus used in each case was equivalent to about 100 mgm. of oven-dry material; somewhat less cell material of the actinomycetes was used. Lead acetate papers were suspended above the solutions.

The cell material of the actinomycetes tended to sink in the medium in some cases (*Actinomyces flavovirens*, *Actinomyces pheochromogenus* and *Actinomyces californicus* on the rhombic sulfur) creating conditions rather unfavorable for rapid sulfide formation. Where the cell substance was in contact with the sulfur on the surface, a slight amount of sulfide was evolved within an hour; similar preparations where the cell material had settled, gave no evidence of sulfide formation in this short time.

None of the uninoculated solutions gave a test for sulfide. With the inoculated solutions, sulfide formation was very general and

TABLE 8

*Sulfide production by heavy inocula of heterotrophic microörganisms in inorganic media containing thiosulfate, or elemental sulfur, or both**

ORGANISM	PERIOD OF INCUBATION				
	1 day	3 days	5 days	7 days	12 days
Thiosulfate medium					
Sarc. lutea	0	0	0	±	±
Az. chroococcum	0	0	0	±	±
B. cereus	0	0	0	0	0
Act. flavovirens	1	4	5	6	8
Act. californicus	0	3	3	3	4
Act. pheochromogenus	0	0	0	0	0
Rhizopus sp.	0	0	±	±	1
Asp. flavus	0	0	0	0	0
Trichoderma sp.	0	0	0	0	0
Thiosulfate with rhombic sulfur					
Sarc. lutea	1	2	2	3	3
Az. chroococcum	0	1	1	2	3
B. cereus	0	0	0	0	±
Act. flavovirens	2	6	8	9	10
Act. californicus	0	4	5	7	8
Act. pheochromogenus	0	2	3	4	6
Rhizopus sp.	1	4	5	6	7
Asp. flavus	±	2	2	3	4
Trichoderma sp.	0	1	1	1	1
Thiosulfate with precipitated sulfur					
Sarc. lutea	1	4	5	6	7
Az. chroococcum	1	2	3	4	5
B. cereus	0	0	0	0	±
Act. flavovirens	3	6	8	10	14
Act. californicus	3	6	8	10	14
Act. pheochromogenus	0	2	3	4	5
Rhizopus sp.	2	4	6	8	10
Asp. flavus	±	2	3	4	5
Trichoderma sp.	1	2	2	2	3
Rhombic sulfur alone					
Sarc. lutea	±	4	5	6	7
Az. chroococcum	0	0	0	0	0
B. cereus	0	0	0	0	0
Act. flavovirens	0	4	8	10	12
Act. californicus	0	4	5	7	10
Act. pheochromogenus	0	±	1	2	3
Rhizopus sp	2	4	5	7	9
Asp. flavus	±	2	2	3	4
Trichoderma sp.	0	0	1	1	1

TABLE 8—*Concluded*

ORGANISM	PERIOD OF INCUBATION				
	1 day	3 days	5 days	7 days	12 days
Precipitated sulfur alone					
Sarc. lutea	1	4	5	6	7
Az. chroococcum	1	1	1	1	2
B. cereus	0	1	1	1	2
Act. flavovirens	3	6	8	12	16
Act. californicus	4	5	8	9	14
Act. pheochromogenus	0	2	3	4	7
Rhizopus sp	2	4	8	9	10
Asp. flavus	±	2	3	4	6
Trichoderma sp	1	2	2	2	2

* 0 = no test; ± = very slight, 1, 2, 3, 4, etc., indicate increasing intensity of dark coloration of the test papers.

more rapid than in the previous experiment (table 8). It is clear that the presence of an abundance of cell material greatly favored hydrogenation of the sulfur. Two of the actinomycetes reduced thiosulfate to sulfide. Strong positive tests for sulfide were obtained with practically all of the actinomycetes and fungi and with some of the bacteria where elemental sulfur was present; stronger tests resulted from the finely divided precipitated sulfur than from the rhombic sulfur. It is probable that the sulfide originated principally from the elemental sulfur, since practically the same amount of sulfide was produced in the media with sulfur alone as in the media containing both sulfur and thiosulfate. The results show that there are quantitative differences in the production of sulfide by the various organisms.

The reaction would probably proceed more rapidly if the cells were mixed with the elemental sulfur in a moist state as a paste, but the object of the present investigation was to determine whether or not various heterotrophic organisms could produce sulfide under the conditions that led to its formation by *T. thioparus* and *T. thiooxidans*, and not to determine differences in the capacities of heterotrophic organisms to hydrogenate sulfur under the most favorable conditions.

None of the solutions gave a test for sulfide with the nitroprusside reagent, nor appreciable change in pH, nor evidence of disappearance of thiosulfate in those cases where it was added to the medium. The amounts of thiosulfate reduced to sulfide by the actinomycetes were therefore extremely slight.

DISCUSSION

These results with heterotrophic microörganisms increase the evidence that hydrogenation of elemental sulfur is effected by many microörganisms and various tissues. During the latter part of the nineteenth century, de Rey-Pailhade (1888a and b, 1898) noted that animal tissues, plant materials, and yeast cells effect this reduction. Heffter (1907) found this reducing capacity inherent in the sulfhydryl groups contained in the organic substances of the cells. He even attempted to measure the protein -SH groups by determining the amount of hydrogen sulfide produced from elemental sulfur through its reaction with these groups converting them to disulfide. More recently, Hopkins (1921) discovered that yeast and various plant and animal tissues contain a compound called glutathione; this was found to be a tripeptide composed of glycine, glutamic acid, and cysteine having a sulfhydryl group which reduces elemental sulfur ($G \cdot SH + HS \cdot G + S = G \cdot S \cdot S \cdot G + H_2S$) (Hopkins, 1929). Callow and Robinson (1925) obtained evidence of the general occurrence of glutathione in bacterial cells. Sluiter (1930) found that the hydrogenation of sulfur by glutathione as well as by tissues was, during a certain time, proportional to the quantity of sulfhydryl present, but many factors determine the number of active -SH groups in tissues at any one time (Guthrie and Wilcoxon, 1932; Mirsky and Anson, 1935-6).

There is much evidence that sulfur is reduced to sulfide by practically all tissues (McCallan and Wilcoxon, 1931). Less is known concerning the mechanism of sulfur reduction by microörganisms, but it has been reported that many of them hydrogenate sulfur.

Miquel in 1879 and Debraye and Legrain in 1890 found that sulfur could be reduced by bacteria. In 1904 Beijerinck noted this for some of the bacteria of the coli-aerogenes group and others

(1904b). Nineteen of 21 bacteria tested by Sasaki and Otsuka (1912) reduced sulfur to sulfide (see also Lederer, 1913 and Tarr, 1933). This reduction by yeast has been known since the studies of Dumas in 1874, and the interest developed by de Rey-Pailhade in what was believed to be a specific sulfur-reducing material in yeast and tissues (see Tanner, 1918, Hopkin, 1921 and Buchanan and Fulmer, 1930, page 201).

Therefore, the hydrogenation of sulfur as effected by *T. thioparus* and *T. thiooxidans* is a common transformation and should have been expected (Buchanan and Fulmer, 1930; Bunker, 1936). The fact that these organisms have this capacity may be interpreted as evidence that their cells contain glutathione or similar compounds containing sulfhydryl groups. It seems most likely that the sulfide produced by these bacteria has the same significance that it has with the numerous heterotrophic organisms which have been shown to effect the same transformation under the conditions used for the sulfur bacteria. In the case of the heterotrophic organisms it was necessary to add more than a small inoculum to obtain an active reaction since conditions in the inorganic media were not well suited for their growth. The sulfur bacteria were able to grow in such media and evolve detectable amounts of sulfide after sufficient cell material had been produced.

Quite recently, van Niel (1936) demonstrated similar reduction of elemental sulfur by purple bacteria. Gaffron (1934–5) believed the sulfide was formed from sulfate (see also Roelofsen, 1934 and 1935) but this did not prove to be the case. The fact that sulfur is hydrogenated by the purple bacteria is evidence that these organisms contain active -SH groups, as do most other microörganisms. Baas-Becking (1925) ascribed more significance to the glutathione and similar materials in the metabolism of the sulfur bacteria, but he assumed that they effected oxidation and not the reduction which has been commonly observed. He believed that hydrogen sulfide, the principal energy source of the higher sulfur bacteria, ionized to form HS- which diffused into the cells, and reacted with cystine or oxidized glutathione, resulting in the formation of elemental sulfur and cysteine or reduced

glutathione. Later results (van Niel, 1931) indicate that the sulfur bacteria are not confined to the use of ionized H_2S and that glutathione might be expected to cause reduction of elemental sulfur rather than oxidation of sulfide in the cells (van Niel, 1936).

One may be inclined to believe that the ability of the sulfur bacteria to utilize elemental sulfur is dependent upon their hydrogenation of the sulfur preceding its entrance into the cells as previously mentioned in this report. This would provide soluble sulfur material. Both *T. thiooxidans* and *T. thioparus* are able to oxidize elemental sulfur, *T. thiooxidans* producing the more rapid oxidation. However, it does not seem necessary to assume that the preliminary hydrogenation occurs.

Primarily, it is difficult to account for the production of sufficient organic matter having sulfhydryl groups to effect the hydrogenation of the sulfur outside of the cells. Presumably these organic compounds would be of use to the cells only in bringing about one reduction reaction. The sulfur would be the single source of energy for the organisms synthesizing these organic materials and all of the other cell constituents. However, the energy required to synthesize two molecules of cysteine would be greater than the energy the bacterium obtains from oxidation of one atom of H_2S to sulfate. It is generally considered that 2 -SH groups are concerned with the reduction of one sulfur atom. Therefore, if the sulfur is reduced before assimilation, some other mechanism than this one must be responsible for the reduction.

It has been shown by McCallan and Wilcoxon (1931), using fungus spores, that elemental sulfur exerts sufficient solution pressure to bring about the diffusion of sulfur into cells which are not even in contact with the solid sulfur particles (see also Sempio, 1932). "Sulphur in the vicinity of fungous spores, by reason of its vapor pressure, gives off sulphur vapor which diffuses into the spores. Here reduction takes place within the spores with hydrogen sulfide as a final product. The reaction is enzymatic in nature and is probably concerned with -SH compounds" (p. 35). If a similar transfer of sulfur takes place with

sulfur bacteria, a mechanism is available whereby the organisms can obtain elemental sulfur directly without requiring preliminary reduction or oxidation. Within the cells, the sulfur would then undergo oxidation characteristic of the specific organisms.

The results of McCallan and Wilcoxon also lead to the conclusion that, at least with the fungus spores, the sulfide is produced inside of the cells after entrance of the sulfur; therefore, evolution of sulfide follows its excretion from the cells. If this is the mechanism of the reaction with the sulfur bacteria, it cannot be assumed that the sulfur becomes available to the cells after preliminary hydrogenation; actually, sulfide production would be evidence that the cells excreted hydrogen sulfide after absorbing elemental sulfur.

Sulfide would be excreted by bacteria only if they were unable to oxidize it. It should be possible to demonstrate sulfide production by purple sulfur bacteria only when the cells are unable to effect their normal oxidations, such as in the absence of light; van Niel (1936) has shown that they reduce sulfur under this condition. With the colorless forms of the higher sulfur bacteria, which produce sulfur globules and do not require radiant energy, it should be possible to demonstrate sulfide formation after inhibiting the normal oxidative processes, as by excluding oxygen.

One might finally conclude that organisms which evolve sulfide under conditions favorable to their normal dehydrogenations, have either little or no capacity for dehydrogenating sulfide or are able to effect unusually active hydrogenation of sulfur; the former condition seems most likely to exist. It is probable therefore, that *T. thiooxidans* and *T. thioparus* are not able to utilize hydrogen sulfide as a source of energy to any appreciable extent under conditions which favor their oxidation of elemental sulfur and thiosulfate (Bunker, 1936).

None of the data gives any indication that the sulfur material produced by *T. thioparus* or the other sulfur bacteria is a polysulfide as conceived by von Deines (1933b), although it is possible that traces of sulfide may be present after having been produced through hydrogenation of the elemental sulfur itself. If the sul-

fide is of importance in the nutrition of the cells or if the sulfur material is a polysulfide, it should be possible to detect it consistently and in appreciable amounts. This is not the case. The sulfur material, as it occurs outside of the cells, has no reducing action on dilute iodine solution and has the characteristics of elemental sulfur.

SUMMARY

The studies were concerned with determining: (1) whether the precipitate, formed by *Thiobacillus thioparus* during oxidation of thiosulfate, is sulfur or a polysulfide; (2) whether or not elemental sulfur is reduced before being absorbed by sulfur bacteria such as *Thiobacillus thiooxidans*.

There was no ash residue after ignition of the material obtained by dissolving the precipitate in carbon bisulfide. The precipitate contained no substance which reacted with iodine solution. Neither sulfide nor sulfite was found in the thiosulfate medium in which *Thiobacillus thioparus* was growing. There was no indication that the precipitate contained sufficient sulfide to characterize it as a polysulfide.

Small amounts of sulfide were evolved by *Thiobacillus thioparus* and *Thiobacillus thiooxidans* growing in solution media. The sulfide is produced through hydrogenation of elemental sulfur.

Various heterotrophic bacteria, actinomycetes, and filamentous fungi likewise evolved sulfide from inorganic media containing elemental sulfur, considerable differences being apparent in the rates of sulfide production by the different organisms. Some of the actinomycetes and fungi produced sulfide from thiosulfate in strictly mineral media.

The hydrogenation of sulfur by the sulfur bacteria suggests the presence of active -SH groups in their cells; this reaction appears to have the same significance with the sulfur bacteria as with the heterotrophic microörganisms.

It is considered unlikely that elemental sulfur undergoes hydrogenation preceding its entrance into the cells of the sulfur bacteria which oxidize the sulfur to sulfate.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

EASTERN PENNSYLVANIA CHAPTER

PHILADELPHIA COUNTY MEDICAL SOCIETY BUILDING, PHILADELPHIA, PA., FEBRUARY 23, 1937

RELATION OF VIRUSES TO NEOPLASTIC DISEASES. *Baldwin Lucké*, University of Pennsylvania, School of Medicine, Department of Pathology, Philadelphia, Pa.

There are a number of viruses which induce multiplication of the host cells which they inhabit; these proliferations may be neoplastic or not, and involve epithelial or connective tissues. Familiar examples of epithelial proliferation not neoplastic in character, are seen in mulloscum contagiosum, fowl-pox and sheep-pox. Of proliferative processes involving connective tissues the best known examples are infectious myxoma of rabbits, and a fibromatous growth described by Shope also occurring in rabbits. It is characteristic of all the virus diseases mentioned to regress. In this respect they differ from true neoplasms.

There are, however, a number of virus diseases with formation of tumors which in both structure and behavior closely resemble neoplasms of man. The first to be discovered was the Rous chicken sarcoma, a malignant tumor usually transmissible by cell-free material, regarded by most observers as of virus origin. To Rous again belongs the distinction of discovering the first malignant mammalian neoplasm of virus origin. He found that the Shope rabbit papilloma may under certain conditions acquire malignant characters and then resemble mammalian carcinoma. The other carcinoma probably of virus nature was discovered by the present writer in the leopard frog. In this species adenocarcinoma of the kidney is of common occurrence; the tumors are invasive and may metastasize.

The fact that several different kinds of tumors in several species of animals are now known to be of virus origin suggests the possible etiologic importance of these agents in many forms of neoplastic disease.

STUDIES IN ACTIVE IMMUNIZATION AGAINST HUMAN INFLUENZA. *Joseph Stokes, Jr.*, The Children's Hospital, Philadelphia, Pa.

STUDIES ON CONCENTRATION AND PRESERVATION OF THE VIRUSES OF POLIOMYELITIS AND INFLUENZA. *H. W. Scherp, I. L. Wolman, E. W. Flosdorf and Mr. D. R. Shaw*, Departments of Pediatrics and Bacteriology, School of Medicine, University of Pennsylvania.

The optimal method has been sought for the preparation and preservation of the influenza virus, for ultimate use in human immunization. At the present time, it is best to cultivate the virus in a medium composed of minced chick embryo and Tyrode's solution. Culture virus is stable for several weeks in

the refrigerator. For longer periods, it is best to dry the culture, in the presence of 5 per cent of gum acacia, using the Flosdorf-Mudd "lyophile" technique.

Purification and concentration of poliomyelitis virus has been studied, using (1) protein precipitation and fractionation methods (2) lipoid extraction methods (3) adsorption and elution methods (4) ultrafiltration (5) vacuum distillation. Best results have been obtained by treating saline extracts of poliomyelitic monkey spinal cords with aluminum hydroxide (Willstaetter's type "C" or Brewer's), eluting the adsorbed virus with disodium phosphate and precipitating the virus by making the eluate 50 per cent saturated with ammonium sulfate at pH 4.8. In this way a highly concentrated preparation may be obtained in a few hours. The virus appears to be unaltered. At least 90 per cent of the lipoid, 90 per cent of the non-protein nitrogen impurities, and 75 per cent of the protein of the original extract are eliminated.

UNSUCCESSFUL ATTEMPTS TO CULTIVATE THE VIRUS OF ACUTE ANTERIOR POLIOMYELITIS IN VARIOUS LIVING CULTURE MEDIA. *John A. Kolmer, Clara Kast and Anna M. Rule*, Research Institute of Cutaneous Medicine, Philadelphia, Pa.

All attempts were made with sterile spinal cord tissue of monkeys developing severe paralysis following intracerebral inoculation with the M.V. strain of virus (Rockefeller Institute). Cultures on the chorio-allantoic membrane of the developing chick embryo by the methods of Woodruff and Goodpasture and Stevenson and Butler were unsuccessful. Cultures in a medium of growing chick embryo in 1:10 dilutions of sterile monkey serum with Tyrode solution in Carrell flasks were unsuccessful. Cultures by the method of Li and Rivers were unsuccessful; monkeys inoculated intracerebrally with the third subculture developed paralysis but this was apparently due to the presence of seed virus without multiplication.

Anaerobic cultures in minced chick embryo in sterile Tyrode solution neopeptone and cysteine hydrochloride were unsuccessful.

ATTEMPTS TO TRANSMIT ACUTE ANTERIOR POLIOMYELITIS TO RABBITS, GUINEA-PIGS, RATS, MICE, CHICKENS AND FERRETS WITH AND WITHOUT DEPRESSION BY X-RAYS. *John A. Kolmer, Clara Kast* and *Anna M. Rule*, Research Institute of Cutaneous Medicine, Philadelphia, Pa.

Young rabbits were not successfully infected with a virulent remote monkey passage strain of poliomyelitis virus by intracerebral, intracisternal, intravenous, intraperitoneal and intranasal inoculation in so far as the production of clinical evidences and spinal cord lesions were concerned. Adult rabbits were not successfully infected by intracerebral inoculation during or after depression by x-rays.

Ferrets were not successfully infected by intracerebral and intraperitoneal inoculations nor by intracerebral inoculation during or after depression by x-rays.

Young guinea-pigs were resistant to intracerebral, intravenous and intraperitoneal inoculations with virus and likewise to intracerebral inoculation during or after depression by x-rays.

White mice were apparently resistant to infection by intracerebral and intraperitoneal inoculation. Preliminary intracerebral injections of sterile starch

did not result in the demonstrable localization of virus injection intraperitoneally.

Chickens were not successfully infected by intracerebral inoculations of virus.

Some mice given repeated intraperitoneal injections of virus, as well as young rabbits inoculated intravenously, succumbed with no demonstrable spinal cord lesions or clinical evidence of poliomyelitis, but the study is being continued to determine the possibility of infection of other tissues and especially of the organs of the reticulo-endothelial system.

MICROÖRGANISMS CAUSING FERMENTATION FLAVORS IN CANE SIRUPS, ESPECIALLY BARBADOS "MOLASSES"[1]

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Received for publication October 4, 1935

During recent years a considerable quantity of a high grade cane sirup has been shipped into this country from the Island of Barbados, British West Indies. This sirup, commonly known as "Barbados Molasses," is sold at a slightly higher price than domestic sirups and is generally distributed in barrels, a comparatively small quantity being sold in retail packages. Upon receipt in this country, the molasses is stored or "cured" in barrels for some months prior to distribution. During this curing period several reactions take place. First there is an active yeast fermentation accompanied by vigorous gassing, during which most of the alcohol is produced. A distinct odor and flavor of alcohol develop. After the fermentation stage, a rum-like flavor develops, and increases in intensity upon prolonged storage.

Browne (1919) in relating his observations of the sugar and sirup industry of Barbados, Tempany (1913) and Watt and Tempany (1905) describe the methods of making "fancy molasses," which is actually sirup. Cane juice, clarified with milk of lime, is boiled down in open tayches. Factories making this type of product maintain a supply of "sour" juice, i.e., cane juice which has undergone natural alcoholic and then acetic fermentations. During the process of concentration sour cane juice is added to invert sufficient of the sucrose to prevent subsequent crystallization, the excess of the volatic acid being boiled away in the tayches. The sirup is evaporated, with continuous skimming, to

[1] Food Research Division Contribution No. 316.

about 36° Baume hot, or about 42° Baume cold. When cooled this sirup is run into puncheons, hauled to port, and stored in underground cisterns, pending shipment. Thus the output from many small factories is blended, overcoming individual variations.[2] Being free from sulphur dioxide, hydro-sulphites, and an excess of lime, these sirups possess a characteristic taste and aroma.

Allen (1906), discussing the manufacture of Jamaica rum from sugar cane products, pointed to the importance of *Bacillus butyricus* and *Bacillus amylobacter* and allied forms in the production of organic acids which are essential in the flavoring of rum. He also described the part played by yeasts in the production of alcohol, a forerunner of the aromatic esters. During the earlier part of the fermentation of the cane juice, members of the *Saccharomycetes* predominated, but as the acidity developed members of the *Schizosaccharomycetes* became prevalent. At the end of the fermentation only bacteria could be isolated from the "dead" liquor. Allen contends that at the end of the yeast fermentation the bacteria utilize the dead yeast cells as a source of food. *Bacillus mesentericus* was thought to produce butyl alcohol in the fermenting mixture. Minute quantities of higher alcohols, furfural, and aldehydes were present, presumably the products of microörganisms.

EXAMINATION OF MOLASSES

Since the changes in the flavor of curing molasses suggest microbial activity, an examination was made of the micro-flora of several samples of Barbados molasses. Also, in order to facilitate further the development of methods for the production of rum flavors in domestic sirups a chemical examination was made of the volatile materials of two barrels of cured Barbados molasses.

ISOLATION AND STUDY OF YEAST

A microscopical examination of sediment (obtained by centrifuging a mixture of equal parts of molasses and water) revealed the presence of numerous yeast and bacterial cells. Several

[2] Information from importer.

attempts to obtain yeast cultures from cured molasses by plating on malt-extract agar and on cane sirup agar[3] were unsuccessful. Finally, tubes of sterile malt-extract broth containing approximately 25 per cent cane sirup were inoculated with the sediment from the molasses. The tubes were incubated at 30°C. for 12 to 18 hours; plates were then poured with cane sirup agar. These plates after six days incubation at 30°C. revealed the presence of a variety of yeast colonies, which were picked and transferred to slants of cane sirup agar, and were later found to be the true agents of the alcoholic fermentation in Barbados molasses.

Eleven cultures were thus obtained from two samples of Barbados molasses made in 1932 and 1933. Cultures from the 1932 molasses were designated by the number 32; those from the 1933 molasses by the number 33. The cultures, maintained on slants of clarified honey agar (Hall and Lothrop, 1934), were studied morphologically, culturally and physiologically. As a result of these studies the yeasts were divided into two groups.

Group I. *Zygosaccharomyces nussbaumeri* Lochhead and Heron. This group includes yeasts numbered 32-1, 32-3, 33-1, 33-5 and 33-6.

The characteristics of this yeast agree essentially with those of *Z. nussbaumeri* Lochhead and Heron (1929). A point of difference is the formation of scum by cultures 32-1, 32-3, and 33-5, but this single difference is not considered sufficient for disagreement with the type species. This yeast was first isolated from honey by Fabian and Quinet (1928) who named it *Z. priorianus* Klocker. Later, however, Lochhead and Heron, discovering an error in Guillermond (1920), renamed it *Z. nussbaumeri*. It has since been isolated from fermented maple sirup by Fabian and Hall (1933).

Group II. *Zygosaccharomyces major* Takahashi and Yukawa. This group includes yeasts numbered 32-2, 32-4, 32-6, 33-2, 33-3 and 33-4.

Since the morphological and cultural characteristics of this

[3] Cane sirup agar was made by adding one part of cane sirup to 2 parts of 2 per cent nutrient agar; the reaction adjusted to pH 6.8 to 7.0 before sterilization.

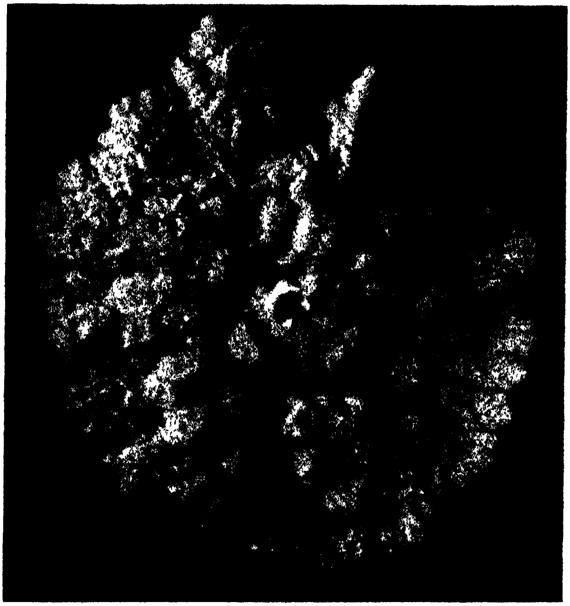

FIG. 1 GIANT COLONY OF YEAST CULTURE 32-1. GROUP I.

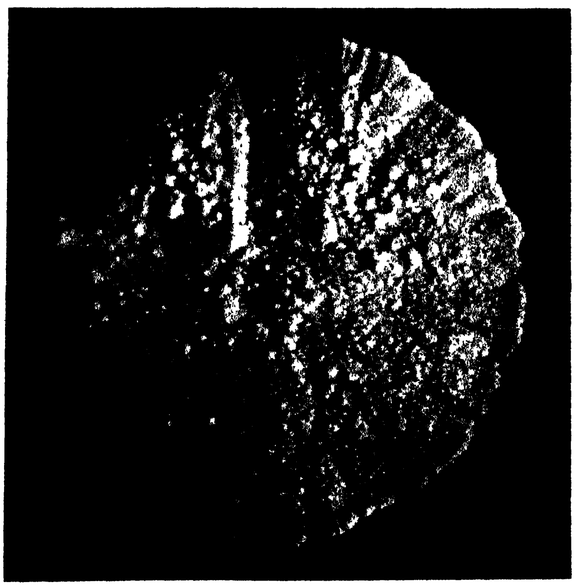

FIG. 2. GIANT COLONY OF YEAST CULTURE 32-4 Group II.

group correspond essentially with those of the yeast isolated by Takahashi and Yukawa from ripening "Shoju" (1912); it is felt that it should be called *Z. major* Takahashi and Yukawa.

The growth in the giant colonies of cultures of Group I (fig. 1) is abundant, and tends towards irregular folds and wrinkles. The edge of the colony is irregular and marked by deep indentations. Gassing frequently occurs within the colony, often causing deep fissures and a crateriform appearance. The growth of giant colonies of cultures of Group II (fig. 2) is moderate, flat and warty. The edge of the colony is irregular, and is free from the warty appearance until after several days of incubation. The color is grayish bordering on brown. The primary growth is dull while the secondary, or warty growth, is glistening.

ISOLATION AND STUDY OF BACTERIA

A study was made of the bacteria, in Barbados molasses, consistently appearing on solid and in liquid media. Plates, made of each sample, were poured with 2 per cent plain nutrient agar, 1 per cent glucose agar, 25 and 50 per cent sucrose agar, and 25 and 50 per cent cane sirup agar, and incubated under aerobic and anaerobic conditions at 20°, 30°, 37° and 55°C. Meat tubes were inoculated from physiological-saline molasses suspensions which had been heated at 80°C. for 10 minutes. To insure a strictly anaerobic condition the meat tubes were heated for 30 minutes in flowing steam prior to inoculation. The inoculated tubes were sealed with autoclaved petrolatum and incubated at 30° and 37°C.

A variety of colonies developed under practically all the test conditions in agar media. No one type of organism predominated, and subsequently none could be found to play a part in the flavoring of sirups. Mold colonies were frequent on plates incubated at lower temperatures under aerobic conditions. The molds proved to be mostly *Penicillium* and *Mucor*. Vigorous gassing and clouding of the broth in all the meat tubes occurred within 24 to 48 hours, so that anaerobic organisms appeared to be most prevalent. Later these were found to play a part in the flavoring of laboratory samples of sirups.

Pure cultures of anaerobic bacteria were obtained by making a

series of shake cultures in yeast-water-agar, which were incubated for 2 or 3 days at 37°C. and then at 30°C. for several days. Although colony development was often slow many well-isolated colonies developed. Subcultures were made from these colonies into tubes of chopped meat medium. All morphological studies were made from meat tubes. Tubes of carbohydrate broths, etc., were inoculated with organisms obtained from the supernatant fluid from the meat tubes.

The growth in the meat-medium tubes was vigorous, accompanied by gassing and a highly putrefactive odor. There was reddening of the meat with subsequent darkening. An examination of cultures revealed a Gram-positive rod occurring singly, in pairs, and in chains. The average length of the cells was 4.5μ the shortest was 3.2μ, and the longest was 5.8μ. The width was from 1.0μ to 1.5μ. No motility was observed. Flagella were not demonstrated. Oval spores were formed and were usually terminal. The rods were not swollen on sporulation.

The organism did not grow on aerobic glucose agar slants but beaded, grayish, viscous colonies appeared under anaerobic conditions. There was no growth on plain agar slants under either condition. In yeast-water-agar stabs a filiform growth appeared after 2 or 3 days along the line of inoculation, followed by fleecy outgrowths extending several millimeters into the medium. Growth occurred in plain broth and was accompanied by a putrefactive odor. Gelatin stabs were completely liquefied in 24 hours at 20°C. Brain medium was blackened. Coagulated albumen was softened. Nitrates were not reduced to nitrites. Indol was produced from tryptophane broth.

Acid and gas were produced from mannose, fructose, glucose, sucrose, maltose and glycerol. Salicin, inulin and galactose were feebly fermented. Plain and litmus milk was completely peptonized after six days incubation at 30°C., gas being produced. This culture is identified as *Clostridium saccharolyticum* Bergey *et al.* Variations from the type species such as motility and spore size were observed. Since all other characteristics correspond so closely with those described for the type culture it does not seem that these differences warrant naming a new species.

When samples of sirup were fermented in the absence of *C. saccharolyticum* the characteristic rum flavor did not develop. That this organism utilizes the residual yeast cells and other organic matter in molasses and produces substances that contribute to the formation of rum flavors seems highly probable.

CHEMICAL EXAMINATION OF VOLATILE MATERIALS

The volatile constituents were distilled with steam from a barrel of cured Barbados molasses. The distillate was fractionated with a Glinsky column, and 2444 cc. of ethyl alcohol boiling below 80°C. were obtained. This alcohol appeared to carry most of the characteristic Barbados flavor. Careful fractionation of it gave a small amount of highly flavored distillate in which furfural was identified by its semi-carbazone. After the removal of the furfural an aldehyde was obtained by treatment with sodium bisulfite. It had an unmistakable odor of vanillin, but chemical proof of the presence of vanillin was lacking. In the nonaldehyde fraction butyl alcohol was recognized by its odor. The constituents forming the true Barbados flavor were not isolated.

A second barrel of Barbados molasses was later distilled, and 15 gallons of distillate were collected. Acetaldehyde came over in the first distillate and was identified by its semicarbazone. Ethyl alcohol boiling at 78.4 to 79.0°C. (2200 cc.) was also obtained from this distillate. The characteristic rum flavor was obtained on fractionating the alcohol and was also obtained from the watery distillates with ether. Hydrolysis or polymerization of the flavor fraction caused great loss in the essential oil which is responsible for the flavor, and only a very small amount was recovered. This had a powerful rum odor. From the flavor fraction and the last runnings of the alcohol, furfural was isolated as its semicarbazone.

The isolation and identification of chemical compounds, which usually result from the metabolism of yeast and bacteria, add additional support to the belief that the flavoring of Barbados molasses is the result of a fermentation similar to that occurring in the manufacture of rum. The chief organism concerned is

necessarily a yeast, upon which reliance must be placed for the production of alcohol. The volatile esters, higher alcohols, furfural and aldehydes probably result from the metabolism of bacteria.

Further evidence to support the theory of rum fermentation in Barbados molasses has been obtained by the development of fermentation flavors in domestic cane sirups. Cultures of the yeast and bacteria previously described have been propagated in samples of high grade cane sirups and have resulted in the production of flavors not unlike those of Barbados molasses.

SUMMARY AND CONCLUSIONS

1. A microbiological examination was made of two samples of Barbados molasses.
2. The yeasts *Zygosaccharomyces nussbaumeri* Lochhead and Heron and *Zygosaccharomyces major* Takahashi and Yukawa were isolated and their rôle determined in the flavoring of cane sirups.
3. Of the bacteria *Clostridium saccharolyticum* Bergey *et al.*, was isolated and probably aids in the flavoring of cane sirups.
4. The volatile substances ethyl alcohol, furfural, acetaldehyde, and butyl alcohol were obtained by distillation.
5. The relationship of yeast and bacteria to the production of rum flavors in domestic cane sirups is suggested.

The authors are indebted to H. S. Paine of the Carbohydrate Division, this bureau, at whose suggestion this work was undertaken and to others of that division, for their many helpful suggestions and coöperative efforts during the course of this work.

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A STUDY OF *d*-ARABINOSE FERMENTATION

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Received for publication December 7, 1936

A comparison of the fermentation of the *d*- and *l*-forms of arabinose has shown that the synthetic *d*-form was fermented slowly and with apparent difficulty by many bacteria which attacked the naturally-occurring *l*-form of the sugar quite promptly (Koser and Saunders, 1932). The results obtained with a number of typical cultures when grown in *d*-arabinose broth at 37°C. are given in table 1. Usually 3 to 10 days were required for fermentation, the time varying with the organism used. Considerable difference was apparent between various strains of the same species. Also in repeated tests with the same culture the time was found to vary somewhat, although within narrower limits. *Proteus* was the only type which was able to bring about a moderately prompt splitting of the sugar. *Proteus* and *Salmonella cholerae-suis* were unusual in that they could effect fermentation of the *d*- but not the *l*-form of arabinose. All of the other types listed in the table fermented the *l*-form promptly. Other organisms, such as staphylococci, streptococci, pneumococci, many of the *Bacillaceae* and yeasts, were unable to ferment either *d*- or *l*-arabinose.

The slow or delayed fermentations which are encountered now and then in bacteriological work have received more attention and study recently, although the explanation of the phenomenon remains largely a matter of conjecture. The late fermentation of lactose by occasional coli-like organisms and the similar slow splitting of lactose and sucrose by certain dysentery bacilli are the best known examples of this sort. In the present report we

[1] This investigation was aided by a grant from the Logan Fund.

record some observations upon the delayed fermentation of *d*-arabinose.

EFFECT OF DIFFERENT CONDITIONS OF CULTIVATION

Concentration of sugar. The effect of increased amounts of *d*-arabinose was studied. A concentrated solution of the sugar was sterilized by filtration and added to broth to give final concentrations of 0.5, 2.0 and 5.0 per cent. The peptone and meat extract of the broth were adjusted to give concentrations of

TABLE 1

The delayed fermentation of d-arabinose

CULTURES	TIME REQUIRED FOR FERMENTATION	CULTURES	TIME REQUIRED FOR FERMENTATION
	days		*days*
Escherichia coli 1	7 to 8	*S. enteritidis* 716	3
E. coli 5	2 to 3	*S. enteritidis* 53	4 to 5
E. coli 18	2 to 3	*S. dysenteriae*, Sonne 268	8
E. aerogenes 2	5 to 7	*S. dysenteriae*, Sonne B	8 to 10
Aerobacter aerogenes 20	9 to 11	*S. cholerae-suis* 666	8 to 9
A. aerogenes M4	7 to 10	*S. cholerae-suis* 667	8 to 9
Klebsiella friedländeri	3 to 4	*S. cholerae-suis* 696	5 to 6
Salmonella schottmülleri 47	8	*S. cholerae-suis* 697	5 to 7
S. schottmülleri 822	5 to 6	*Proteus*, 3 strains	1 to 2

All of the above cultures fermented *l*-arabinose within 24 hours, with the exception of the *S. cholerae-suis* and *Proteus* strains.

Both acid and gas were produced from *d*-arabinose by all of the cultures capable of producing gas from other sugars, with the exception of *S. cholerae-suis*. These strains often produced acid only, or acid with but a small amount of gas.

1.0 and 0.3 per cent, respectively, after addition of the sugar solution. Cultures were inoculated in equal amounts into each lot of medium, incubated at 37°C. and observed closely over a period of two weeks.

With the exception of *Aerobacter aerogenes*, there was very little difference in the time required for fermentation in the several concentrations of sugar. The strains of *Escherichia coli* and representatives of the paratyphoid and dysentery groups produced acid as readily in the 0.5 per cent as in the 5.0 per cent

concentration. In fact, the fermentation appeared at times a trifle earlier in the 0.5 and 2.0 per cent tubes. Several strains of *A. aerogenes*, however, were consistently different in that acid first appeared in the 5.0 per cent concentration and later in the lower concentrations. These results are of interest when compared with the reports of others on the delayed fermentation of lactose by members of the colon group and allied types. Bronfenbrenner and Davis (1918) and Kriebel (1934) have reported that increased concentrations of lactose served to shorten the period required for fermentation.

Oxygen supply. Several reports indicate that slow fermentation of lactose may be accelerated by the increased aeration resulting from a larger surface area in relation to volume of culture. Thus Kennedy, Cummings, and Morrow (1932) observed a shortening of the time necessary to produce acidity in lactose broth when their cultures were grown in toxin flasks. Similar results have been reported by Dulaney and Michelson (1935) who found that the delayed fermentation of lactose by a coli-like organism was accelerated and that "red," or lactose-fermenting, variants appeared earlier in shallow layers of medium. On the other hand, Hershey and Bronfenbrenner (1936) stated that the proportion of fermenting variants in lactose-peptone broth increased more rapidly under conditions of partial anaerobiosis.

In our work, 5 cc. amounts of *d*-arabinose broth containing brom-cresol-purple were distributed into: (*a*) 50 cc. Erlenmeyer flasks, (*b*) ordinary test tubes of 14 mm. inside diameter, and (*c*) narrow test tubes of 10 mm. diameter. In one series of tests 0.5 per cent of sugar was employed and in another 2.0 per cent. Representative cultures of the colon, paratyphoid, and dysentery groups were then inoculated into each of the three types of containers and incubated at 37°C. Relatively little difference was apparent in the time necessary for acid production and the results secured with the narrow tubes and with the flasks were not strikingly different from those seen in the ordinary test tubes. In the shallow layers of medium in flasks there appeared to be an increased production of alkaline products. The acidity following decomposition of 0.5 per cent of *d*-arabinose did not result in as

great a change in pH value as that in the other types of containers. Also the increase in hydrogen-ion concentration under these conditions was quickly followed by a return to the neutral or alkaline range. However, when 2.0 per cent of the sugar was supplied the resulting acidity was more pronounced.

Anaerobiosis. Cultures in one-per-cent *d*-arabinose broth were placed in a jar immediately after inoculation and the usual anaerobic conditions secured by vacuum and the action of pyrogallic acid and sodium carbonate solution. Another series was prepared in which the cultures were inoculated into similar medium under vaseline seal. Under these conditions the delayed fermentation of *d*-arabinose occurred in the usual manner and showed no striking difference from the results obtained with the aerobic incubation in ordinary test tubes.

Addition of d-arabinose to older broth cultures. If sterile *d*-arabinose solution is added to nutrient broth cultures after the cultures have aged for a time equivalent to that required for fermentation, will fermentation of the sugar then be brought about rapidly? In other words, is there any change in the cells while aging in broth which would enable them to make use of the sugar promptly when it is supplied to them later, or must the cells be in contact with the sugar for a time in order to effect the readjustments necessary for its utilization? It is usually assumed that the latter is the correct explanation, although an examination of the literature dealing with delayed fermentation (usually of lactose) has revealed little information upon this point.

Cultures were grown at 37°C. in nutrient broth containing an indicator. Later, sterile *d*-arabinose solution was added to give a concentration of 0.5 per cent. The interval before addition of the sugar to the broth cultures corresponded with the period of delayed fermentation as previously determined for each culture. After addition of the *d*-arabinose solution, the cultures were held at 37°C. and observed daily for evidence of fermentation.

The results were on the whole quite uniform and in every case an additional interval was required before acid or acid and gas appeared. In many cases this interval coincided very closely

with the time required for delayed fermentation when the cultures were grown in the sugar broth medium in the usual manner.

d-Arabinose in synthetic medium. It seemed of interest to determine whether the cultures would be able to develop in a synthetic medium with the *d*-form of the sugar supplied as the only carbon compound. In ordinary nutrient broth containing *d*-arabinose the cells are supplied with numerous available foodstuffs and are not forced to use the sugar in order to attain luxuriant growth. The splitting of the sugar becomes apparent later. In a synthetic medium, however, the sugar could be made to supply the only source of energy and of carbon for structural purposes.

The synthetic medium which was employed consisted of 0.25 per cent ammonium hydrogen phosphates balanced to give pH 6.9, 0.1 per cent potassium chloride, 0.5 per cent sodium chloride, and 0.01 per cent magnesium sulphate. This was tubed in 5 cc. quantities and sterilized in the autoclave. Sterile sugar solution was added to give 0.5 per cent concentration. Two series of tubes were prepared, one containing *d*-arabinose and the other *l*-arabinose as a control. For the tests only those organisms were used which were known to be able to develop in a synthetic medium containing an ammonium salt as the only source of nitrogen and an available sugar as the source of energy.

In the control tubes of synthetic medium containing *l*-arabinose, all strains of *E. coli*, *A. aerogenes*, *Klebsiella friedländeri*, *S. schottmülleri* and *S. enteritidis* developed readily. This would be expected, since these types can utilize this sugar promptly. In contrast to this, most of these organisms failed to develop in the *d*-arabinose synthetic medium. Only one strain of *E. coli* and one of the Friedländer bacillus showed any evidence of multiplication and utilization of the *d*-form of the sugar. The growth in these cases was delayed and scanty. Although growth did not develop in the other tubes, viable cells, as determined by inoculation of agar slants, persisted for some time. Evidently when *d*-arabinose is supplied as the only source of energy, relatively few of the inoculated cells are able to effect the changes necessary

for its utilization and therefore only an occasional culture will develop.

DEVELOPMENT OF STRAINS WHICH FERMENT *d*-ARABINOSE PROMPTLY

Serial transfers in d-arabinose broth. Cultures were inoculated into broth containing 0.5 or 1.0 per cent of *d*-arabinose and an indicator. They were held at 37°C. and observed daily. Transfers were made by the ordinary wire loop to another tube of the same medium as soon as distinct fermentation became apparent. Altogether 26 cultures were carried through serial transplants in this manner.

TABLE 2

Effect of serial transfers in accelerating the slow fermentation of d-arabinose

CULTURES	DAYS REQUIRED FOR DISTINCT FERMENTATION IN EACH SUCCESSIVE TRANSFER
E. coli 1	8, 5, 5, 2, 2, 1+, 1+, 1, 1, 1
A. aerogenes 20	9, 4, 2, 2, 3, 2, 2, 2, 1+, 1, 1, 1
K. friedländeri	4, 1, 1, 1
S. schottmülleri 47	8, 6, 6, 2, 2, 3, 2, 2, 2, 2, 2, 2, 2, 1, 2, 1, 1, 1
S. dysenteriae, Sonne	9, 2, 2, 4, 2, 2, 2, 2, 1, 1, 1
S. cholerae-suis 666	9, 6, 5, 5, 4, 3, 3, 3, 3, 4, 2, 2, 2, 2, 1+ and twenty additional transfers without change

The method proved quite successful in shortening the interval required for fermentation and in most cases it was possible to obtain cultures capable of producing acid, or acid and gas, within twenty-four hours. There was considerable irregularity in the readiness with which the various cultures responded to this manipulation. Some attained this ability after only two or three serial transfers while others required many transfers before an equally prompt utilization of the sugar was evidenced. The behavior of a few representative cultures is shown in table 2.

Cultures of the various organisms which fermented *d*-arabinose promptly were plated directly from the serial transfers onto *d*-arabinose agar containing brom-cresol-purple. Of the resultant colonies, often only a small proportion appeared yellow (acid)

within the first 24 hours while the remainder showed no evidence of acid production at this time. Frequently the proportion of acid-producing colonies was less than 10 per cent of the total and it rarely exceeded 25 per cent. After 2 or 3 days, however, many more of the colonies showed evidence of fermentation of the sugar. Transfers made from the yellow colonies to *d*-arabinose broth gave rise to prompt fermentation, while similar transfers from the bluish-white or non-acid colonies required a longer interval to accomplish splitting of the sugar.

These results show that even though the broth cultures exhibited prompt fermentation they contained cells which varied considerably in their ability to attack the sugar, at least insofar as one may judge from the behavior of the resultant colonies on agar plates. Evidently the serial transfers of fermenting cultures did not eliminate cells which fermented the sugar slowly. There is also the possibility that cells which have acquired the property of speedy fermentation do not uniformly give rise to speedily-fermenting offspring, so that the resulting culture would be mixed with respect to this characteristic.

When cultures of rapidly-fermenting variants were desired for further work they were obtained by serial transfers in *d*-arabinose broth followed by immediate plating on *d*-arabinose-indicator-agar. Colonies which first showed acid production were fished and retested in the sugar broth. This process of enrichment and reisolation of the speediest fermenters was usually repeated several times. As a result cultures were secured which appeared to consist largely or entirely of cells capable of producing prompt fermentation of the *d*-form of the sugar. These strains presented a decided contrast to the original stock cultures from which they had been derived.

Daughter colonies on d-arabinose agar plates. Rapidly fermenting strains could also be obtained from the papillae which appeared in older colonies on *d*-arabinose agar. This phenomenon resembled in general that encountered with the "*E. coli-mutabile*" strains on lactose agar.

When cultures of the coli-aerogenes, paratyphoid or Sonne dysentery types were streaked over plates of nutrient agar con-

taining 0.5 or 1.0 per cent of *d*-arabinose and brom-cresol-purple or other suitable indicator, all colonies were at first white or bluish white in color. When incubation was continued and precautions taken to prevent excessive drying of the agar, small yellowish brown papillae appeared within the colonies. In some cultures these papillae appeared after only a few days, while in others they did not become evident until 10 to 14 days later. At first a few small papillae appeared and they could be detected only with the aid of a hand lens or microscope. Upon further incubation they usually became definitely larger and more numerous until most of the colonies were literally pebbled with them. They were then quite conspicuous to the naked eye. They appeared both in the interior and at the margin of colonies.

When isolations were made from papillae to tubes of *d*-arabinose broth, the resultant cultures produced a much speedier fermentation than the original stock cultures, at times showing definite acid or acid and gas formation within 24 hours. In contrast to this, transfers made from colonies or portions of colonies free from papillae showed the usual delayed fermentation.

It should be added that as a rule the acid end-products of fermentation of *d*-arabinose are not as evident on agar plates as in broth tubes. Usually the plates remained purple (neutral or alkaline) throughout the entire period of incubation even in the presence of thousands of papillae or daughter colonies. The change in color of the indicator was shown only at the papillae themselves. In broth cultures, as already cited, the entire tube became acid.

FERMENTATIVE BEHAVIOR OF VARIANTS.

A number of the quick-fermenting cultures were used for further tests. It seemed of interest to determine whether such cultures, as a result of the "training" in prompt fermentation of the *d*-form of the sugar, might have become altered in their deportment toward the *l*-form or toward other sugars. Accordingly the fermentative abilities of the newly-developed strains were tested with some of the commoner sugars, including several which are not ordinarily utilized. *l*-Arabinose, xylose, rhamnose, glucose, lactose and sucrose were employed for this purpose.

In no instance was any alteration observed. All cultures fermented both the *d*- and *l*-forms of arabinose promptly and there was no change in the deportment toward other sugars. It is perhaps worthy of special note that the slow fermentation of lactose and sucrose characteristic of Sonne dysentery cultures occurred in the usual manner, even though these strains had acquired the ability to ferment *d*-arabinose readily. Evidently the enzymic equipment necessary for the prompt splitting of *d*-arabinose is added to the armament already possessed by the cells without any alteration which can be detected by the usual fermentation tests.

A number of strains of the rapid-fermenters were held for a time to determine whether this newly acquired property would be retained by the cultures for any appreciable length of time. Altogether about 30 cultures were held for periods varying from 10 months to 2 years. After their original isolation from *d*-arabinose serial broth tubes and *d*-arabinose agar plates, they were kept on nutrient agar slants without sugar and transferred about once a month. In the intervals between transfers they were stored in an ice box.

Most of the cultures retained to a surprising degree the ability to ferment the *d*-sugar. Even after a 2-year sojourn on nutrient agar, during which time they had gone through a considerable number of transplants and had not been in contact with the sugar, many of the cultures produced prompt fermentation of the *d*-arabinose. In the majority of cases fermentation was quite distinct in less than 24 hours. When it did not appear or was not pronounced at this time, it usually became evident within 48 hours.

WILL FILTRATES OF ACTIVELY FERMENTING STRAINS ACCELERATE THE FERMENTATION?

It was desired to see whether filtrates of rapidly-fermenting strains would hasten the slow fermentation when added to *d*-arabinose broth. Cultures which produced fermentation promptly were grown in *d*-arabinose broth for periods varying from 16 to 30 hours, at which time they were neutralized with sodium hydroxide solution and passed through Seitz filters. The filtrates were

added in amounts of 0.1, 0.5 and 2.0 cc. to sterile *d*-arabinose broth fermentation tubes. These were incubated 48 hours to test for sterility and then inoculated with stock cultures which gave the usual delayed fermentation. Filtrates of six rapidly fermenting cultures were prepared. Each filtrate was added to a sufficient number of broth tubes so that a series of tests could be made with several additional species as well as with the homologous stock strain.

There was no evidence of any acceleration in the fermentation caused by addition of the filtrates. The interval required for splitting of the sugar was the same as that of control tubes without the filtrate. Thus the presence of a soluble or extracellular "activator" capable of passing through filters was not demonstrated.

SEPARATION OF CELLS AND LIQUID PORTION OF *d*-ARABINOSE BROTH CULTURES AND SUBSEQUENT FERMENTATION

An attempt was made to gain an insight into the course of events by separation of the cells and liquid portion of cultures before fermentation appeared. Cultures were inoculated into 1-per-cent *d*-arabinose broth and incubated for an interval slightly shorter than that required for fermentation. The cultures were then centrifuged to throw down the cells and the liquid portion of the culture was drawn off. In most cases this procedure was applied to cultures about 24 hours before the first evidence of delayed fermentation was expected. That is, if for a certain organism five days were usually required for fermentation, then at the fourth day the culture was centrifuged and the cells separated from the liquid. Just before centrifugation 5 cc. of the culture was removed to a sterile tube, incubated, and the additional time required for fermentation of this part of the original culture was noted. This served to check previous data on the interval necessary for fermentation.

Cells which had been thrown down by centrifugation were immediately taken up and transferred to new tubes of sterile *d*-arabinose broth. In doing this, the volume of new culture to which the cells were added was always the same as that of the

old culture from which the cells had been removed. It will be seen that by this procedure cells which had been in contact with *d*-arabinose in broth for several days were transferred *en masse* and brought into contact with a fresh supply of the sugar before fermentation appeared in the first culture.

In addition to the foregoing procedure, the liquid portion of the culture which remained after centrifugation was immediately put through Seitz filters to remove any remaining organisms and then distributed into sterile test tubes. These tubes of filtrate were then inoculated with the homologous culture, but the cells used for this inoculation were taken from a 24-hour nutrient agar slant of the stock culture and had not previously been in contact with *d*-arabinose. Thus new cells were brought into contact with the sugar after it had been exposed to the effect of the culture for an interval just short of that required for fermentation.

From the foregoing procedures it was hoped to determine whether the cells, during the interval required for delayed fermentation, were changed or adjusted to the sugar (probably in enzymic equipment) in such a way as to enable them to make ready use of the *d*-form of the molecule, or whether instead of such an effect, or perhaps in addition to it, the sugar was being gradually prepared or changed by the cells into a more readily fermentable form.

In table 3 are presented the results obtained on transferring the centrifuged cells to new tubes of *d*-arabinose broth. It will be seen that when cells were removed approximately 24 hours before fermentation and were transferred to new tubes of sugar broth, fermentation in the new tubes occurred promptly, usually within 24 hours. In no instance was there a delayed fermentation corresponding to that ordinarily seen. In a few instances cells were removed 48 hours or more before the usual fermentation. The tests with *A. aerogenes* M4, *S. cholerae-suis* 696 and certain of those with *S. schottmülleri* and *S. dysenteriae* Sonne are examples of this. In general, when cells were removed 2 or 3 days before the usual delayed fermentation, a similar interval elapsed before evident fermentation occurred in the new *d*-arabinose tubes.

Controls were included in all of the foregoing tests by removing

TABLE 3

The effect of transferring cells from d-arabinose broth cultures to new tubes of the medium

CULTURE	TIME REQUIRED FOR FERMENTATION OF d-ARABINOSE*	CELLS REMOVED FROM:	FERMENTATION AFTER TRANSFER OF CELLS TO NEW TUBES OF d-ARABINOSE BROTH
	days		
E. coli 1	6	*d*-arabinose broth at 5th day	Prompt†
		Plain broth at 5th day	Slow, 7 to 8 days
E. coli 1	5	*d*-arabinose broth at 4th day	Prompt
		Plain broth at 4th day	Slow, 5 to 6 days
A. aerogenes 2	6	*d*-arabinose broth at 5th day	Prompt
		Plain broth at 5th day	Slow, 3 days
A. aerogenes M4	7	*d*-arabinose broth at 5th day	In 2 days
		Plain broth at 5th day	Slow, 4 to 5 days
K. friedländeri	3	*d*-arabinose broth at 2nd day	Prompt
		Plain broth at 2nd day	Slow, 2 days
S. schottmülleri 822	5	*d*-arabinose broth at 4th day	Prompt
		Plain broth at 4th day	Slow, 4 to 5 days
S. schottmülleri 822	5	*d*-arabinose broth at 3rd day	In 2 days
		Plain broth at 3rd day	Slow, 4 to 5 days
S. schottmülleri 822	6 to 7	*d*-arabinose broth at 3rd day	Slow, 3 days
		Plain broth at 3rd day	Slow, 6 days
S. enteritidis 53	4	*d*-arabinose broth at 3rd day	Prompt
		Plain broth at 3rd day	Slow, 3 to 4 days
S. dysenteriae, Sonne	7 to 8	*d*-arabinose broth at 6th day	Prompt
		Plain broth at 6th day	Slow, 6 to 7 days

* Determined by separate tests in ordinary fermentation tubes and also by removal of a part of the culture (5 cc.) from large tube just before centrifugation. This 5 cc. was transferred to a sterile test tube, incubated, and the time required for fermentation was noted.

† Prompt fermentation implies distinct acid and gas (or acid only in the case of the Sonne dysentery cultures) within approximately the first day after transfer of the cells. In some experiments this occurred within 16 hours while in others 26 to 30 hours were required.

TABLE 3—*Concluded*

CULTURE	TIME REQUIRED FOR FERMENTATION OF *d*-ARABINOSE*	CELLS REMOVED FROM:	FERMENTATION AFTER TRANSFER OF CELLS TO NEW TUBES OF *d*-ARABINOSE BROTH
	days		
S. dysenteriae, Sonne..	7	*d*-arabinose broth at 5th day	In 2 days
		Plain broth at 5th day	Slow, 5 to 6 days
S. cholerae-suis 667....	7	*d*-arabinose broth at 6th day	Prompt
		Plain broth at 6th day	Slow, 4 to 5 days
S. cholerae-suis 696.. .	6	*d*-arabinose broth at 3rd day	Slow, 2 to 3 days
		Plain broth at 3rd day	Slow, 5 days

cells from cultures of plain nutrient broth at the same time and in the same manner as from the sugar broth. The cells were then transferred to tubes of *d*-arabinose broth. In every case these cells, which had not previously been in contact with the sugar, produced a delayed fermentation. The actual interval required for this effect was at times a little shorter than that normally seen, but it will be recalled that the inoculations were made by transferring cells *en masse* and not by the usual method of carrying over a small inoculum.

One possibility occurred to us which might detract somewhat from the experiments shown in table 3. In the centrifugation and subsequent transfer of cells from older arabinose cultures to new sugar broth tubes, some of the *d*-arabinose from the first tube might have been adsorbed to the cells and thus be carried over to the new tube. While it is unlikely that sufficient sugar would be carried over to account for the vigorous fermentation which was observed in each experiment, nevertheless it seemed best to investigate this point further.

Accordingly several experiments were performed in which the cells, after centrifugation and removal from the first tube of *d*-arabinose broth, were divided into two lots. One lot of cells was added to a proportionate amount of plain nutrient broth without sugar, the other lot was added to new tubes of *d*-arabinose

broth as usual. If sufficient sugar were adsorbed to the cells to account for the prompt fermentation shown in the last column of table 3, then the tubes of plain nutrient broth should become acid.

In no instance did this occur. The nutrient broth tubes which received the cells exhibited pH values of 6.6 to 7.4 at the time (usually 24 hours) when the new *d*-arabinose tubes were distinctly acid, pH 5.5 or less. It was concluded, therefore, that the prompt fermentation obtained upon transferring cells from an older culture was undoubtedly due to actual splitting of the

TABLE 4

The effect of inoculating d-arabinose broth culture filtrates with new cells

CULTURE	TIME REQUIRED FOR FERMENTATION OF *d*-ARABINOSE	*d*-ARABINOSE BROTH CULTURE FILTERED AT:	FERMENTATION AFTER REINOCULATING FILTRATE WITH SAME STRAIN*
	days		
E. coli 1	6	5th day	Slow, 6 to 8 days†
E. coli 5	3	2nd day	Slow, 2+ days
A. aerogenes 2	6	5th day	Slow, 4 to 6 days
A. aerogenes M4	7	5th day	Slow, 11 days
S. schottmülleri 822	5	4th day	Slow, 6 to 10 days
S. enteritidis 53	4	3rd day	Slow, 2 to 3 days
S. dysenteriae, Sonne	7	5th day	Negative throughout

* The cells used for inoculation of the filtrate were taken from 24-hour agar slants prepared from stock cultures. They had not been in contact with *d*-arabinose previously.

† Several tubes were prepared from each lot of filtrate. There was often some difference between these tubes in the time at which fermentation first appeared. This is shown in the table.

newly supplied sugar and not to sugar adsorbed on the cells and carried over from the first culture.

In table 4 experiments are presented wherein the liquid portion of *d*-arabinose broth cultures, after filtration, was reinoculated with cells from agar stock cultures which had not been in contact with the sugar. If any change in the sugar molecule which might facilitate its ultimate breakdown had been brought about as a result of contact with the cells (for an interval just short of that needed for fermentation), then the sugar should be fermented readily by cells from ordinary agar cultures of the same organism.

In no case, however, did this occur. In each instance the fermentation was delayed and the interval was more comparable to that following the ordinary method of inoculation and growth in the sugar broth.

These experiments point to the conclusion that the principal change taking place in the interval before fermentation is an alteration in the fermenting capacity of the cells themselves. When cells are removed after a period of contact with the sugar and are introduced into a new supply of the same, fermentation of this second lot of sugar occurs quite promptly. The process of fermentation by these cells is not appreciably interrupted or delayed by the change in supply of sugar. However, if the cells are taken in a similar way from plain broth (table 3) the usual slow fermentation occurs after placing the cells in sugar broth. Also, the *d*-sugar which has been in contact with cells is apparently not changed in any way which facilitates fermentation, for when the liquid portion of cell-free cultures is inoculated with cells which have not previously been in contact with the sugar, the usual delayed fermentation results.

SUMMARY

A study was made of the delayed fermentation of *d*-arabinose by certain members of the coli-aerogenes, paratyphoid and dysentery groups of bacteria. Increased concentrations of *d*-arabinose in broth, 2.0 per cent and 5.0 per cent, did not accelerate the fermentative process, except in the case of *Aerobacter aerogenes*. The use of shallow layers of medium to afford greater aeration gave no evidence of speedier splitting of the sugar. Anaerobic or partial anaerobic conditions did not appreciably hasten the slow fermentation. In a synthetic medium with *d*-arabinose as the only source of energy most of the organisms were unable to initiate development.

The late fermentation was accelerated by serial passages in *d*-arabinose broth and cultures which had been subjected to this procedure produced fermentation usually within 24 hours. After acquiring ability to ferment rapidly the *d*-form of the sugar, no alteration in behavior toward the *l*-form or toward other common

sugars could be detected. Also, this ability was retained by many cultures for periods up to two years, beyond which the tests were not carried.

On *d*-arabinose agar plates daughter colonies or papillae containing rapidly-fermenting variants gradually appeared within the original non-fermenting colonies.

When cells were removed from *d*-arabinose broth cultures just before fermentation became evident and were transferred to new tubes of the same medium, fermentation appeared promptly. When filtrates of *d*-arabinose broth cultures, from which the cells had been removed just prior to evident fermentation, were reinoculated with new cells which had not previously been in contact with *d*-arabinose, the fermentation was delayed. During the period before evident fermentation the principal change appears to be an alteration in the cells and not a conversion of the *d*-form of the sugar to some more readily assimilable form before it is finally broken down.

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THE INTERMEDIATE DISSIMILATION OF GLUCOSE BY AEROBACTER INDOLOGENES[1]

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Received for publication December 7, 1936

Harden and Walpole (1905) found that glucose fermented by *Aerobacter aerogenes* yielded formic, lactic, acetic and succinic acids, carbon dioxide, hydrogen, ethyl alcohol, 2,3-butylene glycol and acetylmethylcarbinol. Scheffer (1928) has confirmed these as the the only products formed in appreciable quantities. On the basis of carbon balances obtained from analyses of completed fermentations, the latter investigator proposed a breakdown of glucose to methylglyoxal-hydrate which stabilized as lactic acid or split into formic acid and acetaldehyde-hydrate or into pyruvic acid and hydrogen. The pyruvic acid yielded CO_2 and acetaldehyde, the latter with the acetaldehyde-hydrate formed acetylmethylcarbinol or was reduced to ethyl alcohol. 2,3-Butylene glycol was formed by reduction of the carbinol, and formic acid was split into CO_2 and H_2.

The formation of acetylmethylcarbinol and 2,3-butylene glycol from glucose has been of interest as an example of the enzymatic coupling of carbon compounds. Neuberg and Hirsch (1921) demonstrated that acetaldehyde is condensed to acetylmethylcarbinol by yeast when added to fermentations of glucose and presented evidence supporting the suggestion that the condensation involves one molecule of added and one of biologically formed acetaldehyde (Neuberg and Simon, 1933).

The reversion of acidity caused by *Aerobacter* is of interest in connection with the work to be reported. Ayers and Rupp (1918), among others, found that this reversion can take place

[1] Supported in part by Industrial Science Research funds of Iowa State College.

under conditions not dependent upon an alkaline protein decomposition. These investigators were able to show that acetic acid first accumulated in the fermenting medium and subsequently underwent a significant decrease during the fermentation of glucose. Acetic acid, when added to a fermentation of glucose by *Aerobacter*, was partially destroyed. The fate of the converted acetic acid was not determined but they suggested that it was changed to bicarbonate. In contrast with the findings of Ayers and Rupp, Scheffer (1928) found no evidence that *Aerobacter* was able to bring about further conversion of acetic acid.

In the present investigation a study of the dissimilation of glucose has been made by employing serial analysis, a method of attack which Reynolds, McCleskey and Werkman (1934) pointed out to have certain advantages. We have found a destruction of acetic acid with an increase in acetylmethylcarbinol and 2,3-butylene glycol. A general discussion is given of the dissimilation of glucose by *Aerobacter indologenes*.

METHODS

Five-liter fermentations were carried out at 30°C. in 6-liter flasks provided with a delivery tube, permitting aseptic removal of samples, and an exit tube leading to a gas collector.

The medium contained 2.5 per cent glucose, 0.5 per cent sodium bicarbonate and 0.1 per cent dibasic ammonium phosphate. The glucose was sterilized at 15 pounds for fifteen minutes in distilled water. The remainder of the medium containing the buffer was prepared as follows:

The required quantities of bicarbonate and phosphate, with phenolphthalein as indicator, were sterilized in distilled water. Heat sterilization converts the bicarbonate, in part, to carbonate, the conversion being indicated by an alkaline reaction to phenolphthalein after autoclaving. After cooling, the carbonate was reconverted to bicarbonate by forcing sterile carbon dioxide through the solution until the indicator was decolorized. The two solutions were mixed aseptically at the time of inoculation.

The inoculum consisted of a suspension in phosphate buffer (pH = 7.0) of the 24-hour growth from a large agar plate. The

organism, *Aerobacter indologenes*, has been adequately described by Burkey (1928). The culture was examined for purity, microscopically and by plating, before and after completion of fermentation.

Scheffer (1928), on the basis of carbon and oxidation-reduction balances, has suggested that small quantities of succinic acid produced by *Aerobacter* resulted from proteins (yeast and peptone) in the substrate. Under the conditions used in our experiments, no succinic acid could be found. Since the medium contained only ammonium salts as a source of nitrogen, the results support Scheffer's suggestion.

The gases evolved were collected in a graduated container over water saturated with salt and carbon dioxide. Carbon dioxide was determined as the decrease in volume of gas following its absorption in potassium hydroxide contained in a Hempel pipette. Hydrogen was determined by explosion after mixing with oxygen. Residual carbon dioxide in the liquor was determined by acidifying an aliquot portion to congo red with 25-per-cent sulphuric acid, heating and aerating under a small reflux condenser for one hour. The evolved carbon dioxide was collected in an excess of 0.3N barium hydroxide contained in a Brady (1914) absorption tube. The excess base was titrated with 0.3N hydrochloric acid and phenolphthalein.

Glucose was determined by the method of Munson and Walker (1906) on samples previously deproteinated with phosphotungstic acid. Since acetylmethylcarbinol rapidly reduces Fehling's solution it was necessary to make a correction. It has been found in this laboratory that 1 mgm. of acetylmethylcarbinol when treated by the method of Munson and Walker yields 3.14 mgm. of copper oxide.

Acetylmethylcarbinol was determined on separate portions of the fermented liquor according to Stahly and Werkman (1936).

Ethyl alcohol was determined by slowly distilling an aliquot portion of the liquor under a fractionating column until approximately two-thirds had distilled over. The distillate was made alkaline to phenolphthalein and concentrated by one or two successive distillations to a volume of 100 ml. A 50 ml. portion

of the distillate was oxidized by the method of Stahly, Osburn and Werkman (1934) and the resulting acid solution titrated with standard base. Under the conditions of the above oxidation, acetylmethylcarbinol is oxidized to acetic acid, each mole of carbinol giving two moles of acid. Since an appreciable quantity of the carbinol present distills over, under the conditions described, it is necessary to make the determination on a separate portion of the distillate and apply a correction. Recovery of alcohol by the above oxidation method is 94 per cent. The total

TABLE 1

Dissimilation of glucose by Aerobacter indologenes

Glucose fermented and products in millimoles per liter

TIME	GLUCOSE FERMENTED	HYDROGEN	CARBON DIOXIDE	FORMIC ACID	ETHYL ALCOHOL	ACETIC ACID	LACTIC ACID	ACETYL METHYL CARBINOL	2,3-BUTYLENE GLYCOL	C RECOVERY	REDOX INDEX*
hours										*per cent*	
24	14.33	0	7.12	21.0	16 70	6.00	0.55	0 226	6 02	116.3	0 68
28	32.70	0.725	24 15	—	21.30	—	1.00	0 452	13.87	—	—
31	36.10	2 26	34.35	24.0	22.70	8 65	1.47	0.78	16.70	90.5	0 935
35	41 00	3.44	46.50	30.0	30.65	6 80	3.29	0 452	22.30	102 5	0.932
47	61.10	7 07	85.60	33.2	43.00	5 00	3 90	1.22	35.45	102 0	1 013
64	82.50	17.32	110.50	31.75	55.20	2.90	4 13	0 452	49 80	95.3	0 909
81	100 30	23.95	144 30	34.60	68.30	1.70	4 02	0 11	66 05	99 0	0 90
110	123.80	34 20	189 0	34 60	82 50	1 10	3 68	0 89	79 40	97.0	0.943
209	127 30	45.00	218.3	21.60	88.30	0 60	3 68	0	84 25	100 3	0 956

* Cf. Erb, Wood and Werkman. J. Bact. **31**, 595 (1936). A perfect balance $\left(\frac{\text{Oxidized}}{\text{Reduced}}\right)$ gives an index = 1.0.

acid minus that due to acetylmethylcarbinol divided by 0.94 gives the acid equivalent of the alcohol present in the distillate. Only ethyl alcohol was present.

2,3-Butylene glycol was determined by the method of Brockmann and Werkman (1933). Unfermented sugar was removed by copper-lime precipitation (Hewitt, 1931) before carrying out the alkaline distillation. Failure to do so results in caramelization of the sugar, giving rise to compounds which interfere with the determination of the glycol as well as with that of lactic acid.

Lactic acid was determined by the method of Friedemann and

Kendall (1929) on the residue remaining from the 2,3-butylene glycol distillation.

The combined residues remaining after the previously described distillation for ethyl alcohol were acidified to congo red and steam-distilled according to the method of Olmstead, Whitaker and Duden (1929). Volatile acids in the distillate were determined as described by Osburn, Wood and Werkman (1933). It was found that acetylmethylcarbinol does not interfere with their mercuric oxide oxidation method for the determination of formic acid. Only formic and acetic acids were present.

EXPERIMENTAL

Periodic analysis of the fermentation of glucose by *Aerobacter indologenes* (table 1) reveals that three products, i.e., formic acid, acetic acid and acetylmethylcarbinol undergo such decreases following previous accumulation as to be assigned significant rôles as intermediates.

It is evident that if the biological conversion of a sugar such as glucose into final products occurs in such manner that the sugar and its decomposition products can be equated chemically throughout the fermentation, the following relationships hold: each mole of glucose disappearing must be accompanied by the appearance of a definite number of millimoles of a given initial product, so that, graphically, the relationship between product and converted substrate is linear, assuming no change in the character of the fermentation. This linearity should hold except where a product is subject to further conversion, such as the decomposition of formic acid to carbon dioxide and hydrogen when the sum of carbon dioxide (or hydrogen) and formic acid should give the linear relationship. With these considerations in mind it may be possible to gain some conception as to the fate of those products showing characteristics of intermediates.

In figure 1, ethyl alcohol, carbon dioxide, formic acid and the sum of formic acid and carbon dioxide have been plotted against glucose fermented. The curve for ethyl alcohol is linear throughout. The sum of carbon dioxide and formic acid likewise shows the linear relationship, showing an intimate connection between

carbon dioxide and formic acid and indicating that carbon dioxide is formed by decomposition of the intermediately formed acid.

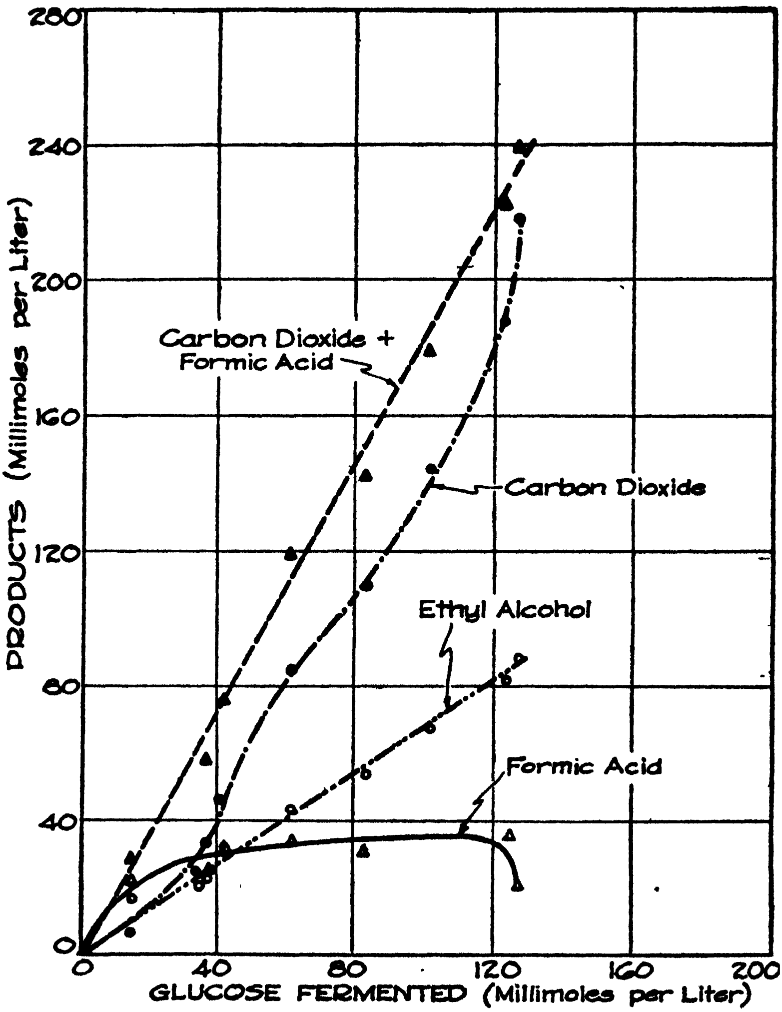


FIG. 1

If the decrease in acetic acid (table 1) be attributed to its conversion to one of the above products, we should expect the linear relationship to be destroyed, unless the change, for example, to ethyl alcohol be accompanied by a simultaneous and equivalent

decrease in the formation of the alcohol from glucose. Occurrence of the latter phenomenon is highly improbable.

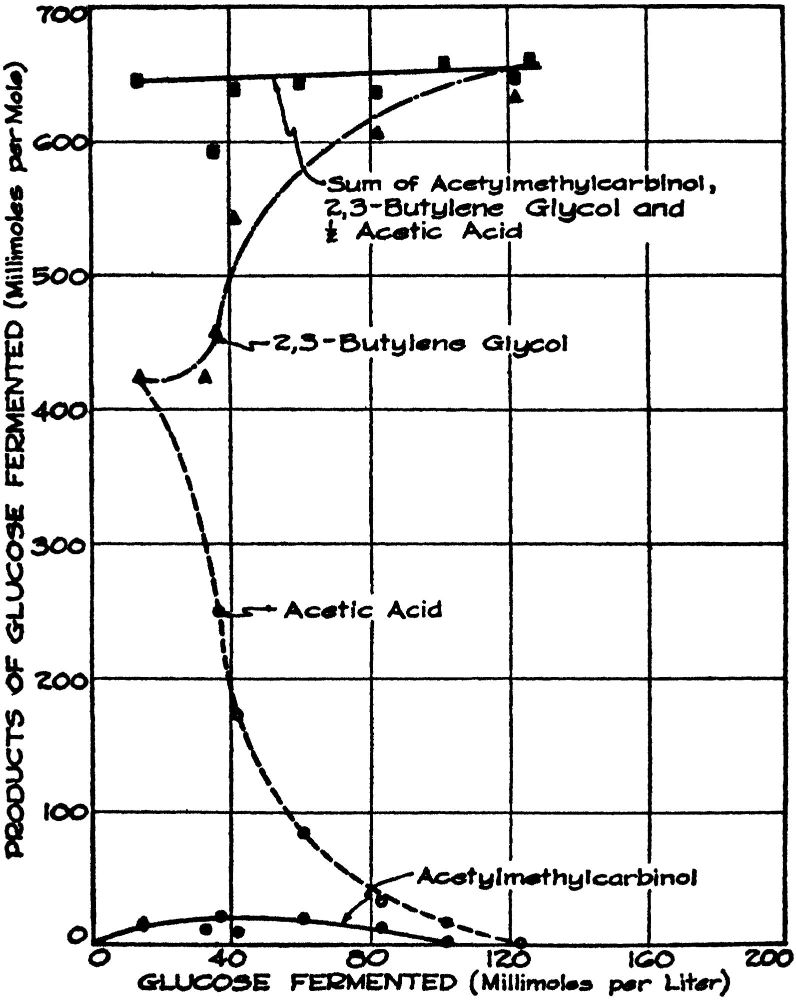


FIG. 2

With the small quantities of lactic acid, there remain only acetylmethylcarbinol and 2,3-butylene glycol to account for the converted acetic acid. In figure 2, these products have been plotted as millimoles per mole of glucose fermented against milli-

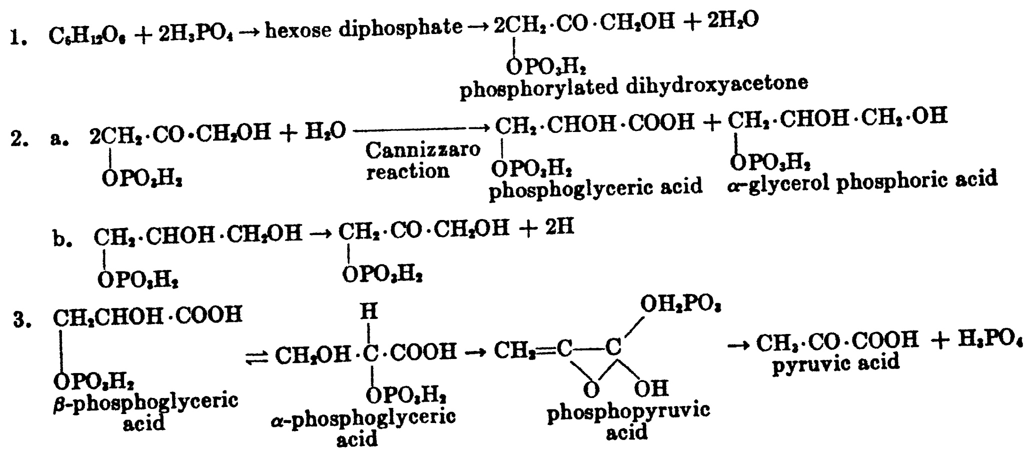
moles of fermented glucose. It will be seen that a constant rate of formation is indicated by a straight line parallel with the abscissa. The curves for 2,3-butylene glycol and acetic acid show a reciprocal relationship while a graph of the sum of acetylmethylcarbinol, 2,3-butylene glycol and one-half the acetic acid is approximately linear. The data indicate that the acetic acid intermediately formed, is condensed and reduced to 2,3-butylene glycol.

The suggested conversion of acetic acid to 2,3-butylene glycol probably takes place through preliminary reduction of the acid to acetaldehyde, followed by condensation of the latter to acetylmethylcarbinol and subsequent reduction of the latter to 2,3-butylene glycol. The catalytic reduction of acetic acid to acetaldehyde is well known in organic chemistry. That acids can be reduced biologically by bacteria has been adequately demonstrated for the butyl forms by Reilly *et al.* (1920). The biological condensation of aldehydes to carbinols has already been mentioned. Condensation of acetaldehyde to acetylmethylcarbinol by ultraviolet radiation without intervention of biological systems has been demonstrated by Dirscherl (1931) and by Barak and Style (1935). The reduction of acetylmethylcarbinol to 2,3-butylene glycol by *Aerobacillus* and butter cultures has been shown by Hammer, Stahly, Werkman and Michaelian (1935). The suggested conversion is consistent with established chemical and biological principles.

Neuberg, Nord and Wolff (1920) have reported the fixation of acetaldehyde in the fermentation of glucose by *Aerobacter* and the conversion of acetaldehyde to equal molar mixtures of acetic acid and ethyl alcohol by the same organism. (Neuberg and Windisch, 1925.) We have identified both acetaldehyde and pyruvic acid as fixation products in the fermentation. In 1914, experimental results led Neuberg to suggest the bacterial decomposition of pyruvic acid according to the equation:

$$CH_3 \cdot CO \cdot COOH + HOH = CH_3COOH + HCOOH$$

Recently we have reported the isolation of phosphoglyceric acid formed by colon-aerogenes organisms from glucose (Werk-



4. a. $CH_3 \cdot CO \cdot COOH + HOH \rightarrow CH_3 \cdot COOH + HCOOH$
   b. $CH_3 \cdot CO \cdot COOH \rightarrow CH_3 \cdot CHO + CO_2$

5. a. $CH_3 \cdot CHO + 2H \rightarrow CH_3 \cdot CH_2OH$
   b. $CH_3 \cdot COOH + 2H \rightarrow CH_3 \cdot CHO + H_2O$
   c. $CH_3 \cdot CO \cdot COOH + 2H \rightarrow CH_3 \cdot CHOH \cdot COOH$
   d. $CH_3 \cdot CO \cdot CHOH \cdot CH_3 + 2H \rightarrow CH_3 \cdot CHOH \cdot CHOH \cdot CH_3$

6. $2CH_3CHO \rightarrow CH_3 \cdot CHOH \cdot CO \cdot CH_3$

7. $HCOOH \rightarrow H_2 + CO_2$

Fig. 3

man *et al.* 1936). These findings, in addition to recent investigations pointing to the phosphorylated trioses as primary intermediates in the biological decomposition of hexoses (cf. Lohmann, 1930; Harden, 1933; Embden, Deuticke and Kraft, 1933; Meyerhof, 1933; Meyerhof and Kiessling, 1933; 1935, Tikka, 1935) suggest that the mechanism of muscle glycolysis of Embden and Meyerhof has its counterpart among bacteria.

In figure 3 is given a series of reactions assumed to occur in the dissimilation of glucose by bacteria of the genus *Aerobacter* as determined with a typical species, *Aerobacter indologenes*.

Acetic and pyruvic acids, acetaldehyde and acetylmethylcarbinol will serve as hydrogen acceptors for the oxidation of the intermediate triose (dioxyacetone-phosphate, glycerolphosphoric acid?). Since formic acid is decomposed and the ratio of hydrogen to carbon dioxide becomes progressively less than one, some or all of the reductions of equations under 5 can take place with utilization of hydrogen liberated by reaction 7.

The previously mentioned linear relationship between glucose fermented and the sum of formic acid and carbon dioxide suggests that intermediately formed formic acid is changed in accordance with reaction 7. Since, however, the same linearity will occur in the presence of 4b, if the latter proceeds at a uniform rate, the possibility that pyruvic acid is converted in both ways cannot be excluded. Unpublished results show that no hydrogen is evolved when glucose is fermented by *A. indologenes* in the presence of an excess of acetic which is reduced according to 5b. Evidence failed to indicate further conversion of acetic acid as in the Thunberg-Wieland (Neuberg and Simon, 1933b) series, while the quantitative relationship between the acid and 2,3-butylene glycol support the previously suggested reduction and condensation of the acid to the glycol.

SUMMARY

A series of reactions is proposed to account for the dissimilation of glucose by organisms of the genus *Aerobacter*.

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THE INACTIVATION OF ENCEPHALITIS VIRUS (ST. LOUIS TYPE) BY MEANS OF SOFT X-RAYS

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Received for publication December 7, 1936

Although it is well known that bacteria, yeasts, and molds can be killed by irradiation with soft x-rays, little work has been reported concerning analogous work on virus suspensions. Bruynoghe and LeFevere de Arric (1925) inactivated several viruses using the radiations from radium, while Lenz and Jungeblut (1932) reported no inactivation using hard x-rays. The lack of inactivation in this latter case may have been due to the fact that with the penetrating hard x-rays not enough of the radiant energy was absorbed by the virus or that the period of exposure was too short to absorb enough energy to cause inactivation. If it is this absorbed energy which causes inactivation, then one would expect better results with x-rays in the "soft" or long wave length region, since this type of ray is more readily absorbed. With this in mind the experiments described below were carried out using radiations of longer wave-length for longer periods of time than has been heretofore reported in the case of viruses.

METHODS AND APPARATUS

The entire irradiation technique was the same as that described by the authors (Moore and Kersten, 1936) in a previous publication. Briefly, it consists in placing the material to be irradiated in a small glass dish, 10 mm. deep and 15 mm. wide and placing the dish at a distance of 5 cm. from the focal spot of a water-cooled x-ray tube. With this technique it has been demonstrated that the temperature of the suspension being irradiated does not rise more than 3°C. above room temperature which rules out the possibility of inactivation by heat.

Mouse brain tissue, either glycerinated or fresh, was ground in a sterile mortar, transferred to the irradiation dishes, weighed and irradiated for four hours. After irradiation the brain tissue was transferred quantitatively to sterile tubes and made up to a concentration of 10 per cent based on the original weight of the sample. Either Tyrode's solution or saline was used as the diluent. The water-clear suspensions were prepared by centrifuging a 10 per cent crude suspension of brain tissue at about 2000 r.p.m. for 30 minutes and then irradiating the supernatant for 4 hours. The glycerinated tissue used was never older than 10 days and the fresh tissue was removed from the animal and used at once.

The inoculations were intracerebral (0.03 cc.) into Swiss mice weighing from 15 to 25 grams. Control animals received an equal dose of suspension prepared from brain tissue which had been kept next to the x-ray tube during the irradiation period in order to insure that temperature effects would be nearly the same.

SUMMARY

The results of 7 experiments in which 59 animals were used are summarized in tables 1 and 2. The failure of experiments 1 and 2 may be attributed to the fact that the depth of the 2 cc. of material in the irradiation dish was too great to permit the rays to penetrate with sufficient intensity to the lower layers. In these 2 experiments a total of 22 animals were used, and although they all died with typical symptoms, the average incubation period of the disease was 9 days. The control animals were 4 in number and all succumbed with typical symptoms at the end of 3 to 5 days.

In the later experiments, numbers 3, 4, 5, 6, and 7, the depth of the material was one-half that used in the first 2 experiments and the experimental animals all survived and were reinfected about 3 weeks later. They all then succumbed, indicating that there was no immunity from the previous injection and, therefore, unlikely that living virus was present in the original injection.

Experiments 6 and 7 were performed to rule out the possibility of the virus being effected by decomposition products of the brain tissue itself or the small amount of glycerin which may have adhered to the brain tissue in spite of thorough washing, or possible decomposition of the Tyrode's solution. Experiment 6 utilized glycerinated tissue suspended in saline and then centrifuged to remove all excess tissue. The clear supernatant liquid was then irradiated. Experiment 7 used fresh tissue suspended in saline and then centrifuged to remove all excess tissue. In both cases all the experimental animals survived and all the controls succumbed, thus demonstrating that decomposition products were not a contributing factor.

TABLE 1

Showing the effects of 4 hours irradiation of encephalitis virus with soft x-rays (λ = *1.37A–1.54Å*)

EXPERIMENT NUMBER	NUMBER OF EXPERIMENTAL MICE	NUMBER OF EXPERIMENTAL ANIMALS SURVIVING	NUMBER OF CONTROL MICE	NUMBER OF CONTROL MICE SURVIVING	AMOUNT OF MATERIAL IRRADIATED	KIND OF TISSUE
					cc.	
1	12	0	2	0	About 2	Fresh
2	10	0	2	0	About 2	Glycerinated
3	6	6	2	0	1	Glycerinated
4	8	7*	4	0	1	Glycerinated
5	7	7	3	0	1	Glycerinated

* One of the 8 experimental animals died of infection. A coccus cultured from the heart's blood produced death in a susceptible animal.

TABLE 2

Showing the effect of 4 hours irradiation of purified encephalitis virus suspension with soft x-rays (λ = *1.37A–1.54Å*)

EXPERIMENT NUMBER	NUMBER OF EXPERIMENTAL MICE	NUMBER OF EXPERIMENTAL ANIMALS SURVIVING	NUMBER OF CONTROL MICE	NUMBER OF CONTROL MICE SURVIVING	AMOUNT OF MATERIAL IRRADIATED	KIND OF TISSUE
					cc.	
6	8	8	3	0	1	Glycerinated
7	8	8	3	0	1	Fresh

CONCLUSION

Encephalitis virus (St. Louis type) has been inactivated by irradiating virus containing tissue and purified water-clear suspensions with soft x-rays for 4 hours. The inactivation has been demonstrated by inoculating susceptible animals and subsequently killing the same animals with inoculation of active virus.

The writers wish to express their appreciation to Dr. Maurice Brodie for helpful suggestions in this work.

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THE INSECT MENACE IN THE BACTERIOLOGY LABORATORY

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Received for publication January 13, 1937

At a recent gathering of bacteriologists from all parts of the country a complaint frequently heard concerned the increasing number of molds in the bacteriology laboratory, and the difficulty in controlling them. The possibility that insects play a rôle in the spread of molds seems not to be generally recognized. An experienced bacteriologist has remarked, "Very old cultures almost always become contaminated with molds unless they are sealed from the air. I think the spores are drawn in from the air as the agar and plug shrink from drying." A bacteriologist working in another city has told the writer of finding some of his most carefully cherished plate cultures alive with mites. Puntoni (1931a) reports that an apparent return to virulence in BCG cultures was found to be due to a contamination by virulent tubercle bacilli carried from near-by cultures by mites. Very seldom have mites themselves been found in the author's own cultures of bacteria; but experience has taught that when molds begin to appear prompt measures should be taken to check mites. These crawl between cover and dish of agar plates, find an uncongenial environment, and usually do not stay. Later marginal colonies of a mold or of a foreign bacterium develop in their wake. In mold-contaminated test-tube cultures a careful examination with the hand lens often reveals parallel lines of minute bacterial colonies beyond the range of growth from the original points of inoculation. These have developed along the lines of progress of the mites as they have wandered over the culture carrying bacteria on their tarsi.

Mites have long been well known to Drosophilists, to many

mycologists, and to some bacteriologists. Erwin F. Smith (1920) warned against them, and Thom (1930) describes their devastating activities and suggests general precautions. In mold cultures these insects[1] may feed and breed under cover of the thick mycelium and may not be discovered unless they are purposefully sought with a low microscopic magnification and adequate illumination. They will travel from culture to culture carrying conidia and bacteria on the surface of their bodies and leaving them in their wake. On plates as well as in tube cultures, if they walk over agar where growth has not as yet developed, parallel lines of small evenly-spaced bacterial colonies may develop following their path of progress. Mold colonies appear in far less abundance; the conidia are much larger than the bacteria, they are carried on other parts of the body beside the tarsi, and come into contact with the agar less often. Also, conidia frequently require more moisture for germination than do bacteria for their growth.

Certain facts concerning the habits and life history of mites explain some of the difficulties encountered in their control. Many if not most of the mites which are laboratory pests belong among the *Tyroglyphidae*. Banks (1915) states that at one period of their development the young Tyroglyphs develop a plate of sucking discs by means of which they attach themselves to an insect, and in a non-motile and non-feeding form, the hypopus, are transported to a new feeding ground. Thus, cockroaches and flies in a laboratory are potential foci for the spread of mites.

Fortunately for bacteriologists, not all laboratories are located in centers where cockroaches abound. However, with the importation of glassware directly from such centers there is a recurring possibility of the introduction of these insects with the excelsior and paper wrappings of the shipments. Many bacteriologists are not familiar with the annoyance which roaches themselves cause in plate cultures. Young roaches, attracted by the agar, will enter a petri dish and wander over the surface of the culture, bringing mold conidia and bacteria with them from the dust of the room. Some of them, at least, either cannot exert strength

[1] Mites are popularly, but incorrectly, called insects. Even a good medical dictionary so defines them.

enough to force their way out again, or do not care to do so, and hence they may be found actively exploring all parts of the agar surface. Several years ago, while engaged in hospital work, we found a three-quarter inch roach in a virulent *Eberthella typhi* plate. We have never caught a full grown roach in a culture plate, though part of an egg case was once recovered from the surface of the agar in such a dish.

Far more insidious than the cockroach, and perhaps often traceable to its presence, is the large group of microscopic mites. On old wood such as window sills, cupboards, chairs, floors, sharp scrutiny often reveals rapidly moving animals so minute as ordinarily to pass unnoticed. These are sometimes called "wood lice," and in their inconspicuous yet numerous existence they are typical of the *Acarina.* In the course of time these wood mites can crawl into a petri dish culture, leaving mold or bacterial spores to develop into colonies later. The *Acarina* are small in size. The imagoes may be more or less than 0.5 mm. in length; they have *four* pairs of legs. The larvae are so small as to be altogether invisible without a strong hand lens or microscope; they have but *three* pairs of legs. The several genera are almost omnipresent, and their habits of life make it possible for them to penetrate into locations which are wont to be considered safe from any kind of infestation. Some of them, as the "itch mite," are generally familiar by reputation. Yet not as parasites, but as vectors, do mites need consideration in a bacteriological laboratory. As mechanical carriers of common dust organisms, the familiar mold and bacterial "air contaminants" which appear in laboratory cultures, these insect invaders require certain precautions on the part of the laboratorians.

The writer's own knowledge of mites as a cause of contamination in bacterial cultures came about as the result of a growing interest in actinomyces and hyphomycetes, and the gradual accumulation of pure cultures of such forms. During a summer vacation mites found their way into a "dust-proof" cabinet in which all the stock bacterial and mold cultures had been stored, and travelled from tube to tube sowing actinomyces and molds and foreign bacteria in most tubes. The initial work of plating and fishing which was necessary to reestablish pure cultures was

slight in comparison with subsequent efforts to eradicate the mites from the storage cabinets and their environs.

No perfectly satisfactory method of combating mites has been found, but certain measures have proved to be of sufficient value to warrant endorsement. First among these is the exclusion from the laboratory of everything from outside environments, especially insects. Flies carry mites from plants and plant refuse such as rotting fruits. Fine-meshed window screens and a spray gun are used for them. Sodium fluoride mixed with powdered sugar is an efficient roach control agent but its danger to laboratory cleaners makes its use questionable.[2] Various powders on the market have value for this purpose; they must be used around every water pipe, at the base of every piece of furniture, and around the edges of the room, and must be renewed every ten days. None of those which we have tried has been very satisfactory. Sprays also are used against cockroaches. One which is marketed under the trade name of Kil-in-Sec[3] has proved to be especially effective.

The use of metal instead of wood in laboratory furnishings, and frequent and free use of disinfecting solutions, such as lysol, in dusting the entire laboratory are of very real value, though of a general rather than of a specific nature. A 2 per cent solution of lysol will kill some mites only if they are immersed in it for several minutes. Experiments have proved that their surface is difficult to wet, and that they are very resistant to chemicals.

Bacteriological techniques modified for use against locomotive, free-living invisible forms should be more successful than they have proven. It is hard for a bacteriologist to orient himself to the presence of organisms which pass cotton filters in both directions. Mites pass easily between the fibers of firm cotton plugs, travelling from tube to tube through a whole basket of cultures, mixing organisms throughout their progress. This has occurred even in cultures capped with tinfoil which was tightly bound down with rubber bands. Thom (1930) and Puntoni (1931b) recom-

[2] If sodium fluoride enters a break in the epidermis it causes a progressive death of the tissue which may result in the necessity to amputate. Its action can be checked by applying strong carbolic acid, and then neutralizing with alcohol.

[3] Kil-in-Sec is manufactured by the Capitol Chemical Co., Washington, D. C.

mend the use of poisoned plugs to keep mites from entering test tube cultures of molds. Our own experience in applying this technique to bacterial cultures has been so disastrous that we strongly advise against it for this group of organisms. Other means of preventing migrations must be sought.

Three procedures have proved especially effective in mite control. First, shelves were built with legs which, protected by glass cups, were stood in broad shallow basins of lysol solution.[4] The whole was placed on a table several inches from the wall of the room. Shelves and framework were thoroughly brushed with a mixture of equal parts of turpentine and linseed oil. This killed all insects and mites on the wood. These shelves are kept wholly for the care of cultures. All equipment for use in stock transfers is brought directly from the autoclave to these shelves, thus insuring a clean foundation for work. It has been found that strong acids killed instantly those species which infested our cultures,[5] though no other types of chemical accomplished this. The next procedure, therefore, is to remove items from these shelves only to such surfaces as have been freshly wiped with 50 per cent acetic acid solution, or to iron tripods which have been freshly flamed. Plate or test tube cultures are wiped with 50 per cent acetic after study, or are treated with dichlorbenzene as described below, before being returned to the shelves. This is not so sure an agent as the acetic acid, but it is less disagreeable to the worker. Hands are thoroughly washed and vigorously rubbed before handling any of the cultures.

It has been found that para-dichlorbenzene effects the destruction of living mites rather quickly, though it does not destroy their eggs. So far we have not found it injurious to yeast, mold, or bacterial cultures, though not all species have been exposed to it. The third procedure therefore is to place mite-infested or suspected cultures in a covered jar which contains an open dish of dichlorbenzene crystals. The caps of culture tubes are

[4] Erwin F. Smith used mercuric chloride, 1:1000, and considered that the fine crystals after evaporation were equally as effective as the solution. We do not find them a deterrant to cockroaches which in turn may carry mites.

[5] Most of our experimental work has been done on *Tyroglyphus lintneri*, which proved to be most persistent in fungus cultures. *Tyroglyphus* and *Tarsonema* are the two genera which probably do much of the laboratory damage.

loosened to allow free passage of gases. One hour of this treatment has been found ample to kill mites in petri dishes; so two hours have been adopted as the routine duration of treatment for petri dish, and four hours for test tube cultures. This process is repeated every forty-eight hours for a period of twelve days,[6] to insure the destruction of the larvae as they hatch from the eggs. Open dishes of dichlorbenzene are closed into cupboards and closets and incubators where infestation is suspected, and the two-hour exposure to the crystals is repeated at forty-eight hour intervals as above, as lysol does not eradicate mites from shelves or cupboards.[7]

The procedures outlined above are of great assistance in controlling the activities of mites, but they cannot be considered as 100 per cent effective. Experienced mycologists and Drosophilists alike agree that eternal vigilance is the price that must be paid for freedom from the depredations of mites. Though bacteriologists have at their disposal a technique developed for dealing with invisible organisms, they must enlarge its scope to include this group of locomotive forms which can pass through cotton filters, and multiply in dry environments as well as flourish in well moistened air. Especially should this be done in the face of the growing interest in variation and in cyclogenic studies, where the most rigid conditions for pure culture perpetuation must be maintained.

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[6] Eales (1917) reports that *Tyroglyphus longior* eggs requıre ten to twelve days to hatch.

[7] Since dichlorbenzene is somewhat irritating to man it should be kept in closed spaces.

STUDIES UPON MINUTE HEMOLYTIC STREPTOCOCCI

III. Serological Differentiation[1]

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Received for publication January 20, 1937

In previous papers (Long and Bliss, 1934; Long, Bliss and Walcott, 1934) the cultural characteristics and distribution of minute hemolytic streptococci were discussed. These may be summarized as follows: Minute hemolytic streptococci produce extremely small colonies, ranging in size, in poured blood agar plates, from complete invisibility to 0.13 mm. in diameter after 18 hours and from 0.14 to 0.40 mm. after 48 hours growth. The zone of hemolysis, which is of the *beta* type, may be from 2½ to 18 times as large as the colony. The organisms, as observed in smears, are about two-thirds the size of ordinary *beta* streptococci. They tend to lie in clumps, although moderately long chains also are present. They are amphophilic to the Gram stain. They are easily grown in blood broth and blood agar and in plain broth containing as little as 0.075 per cent glucose. Cultures in blood broth under a vaseline seal have been found to be viable after a year in the refrigerator. Most of the strains were isolated from throat cultures, some from normal individuals, more from patients suffering from nephritis and rheumatic infections. We are indebted to Dr. Rebecca C. Lancefield and to Drs. Beatrice and David Seegal for a number of strains.[2] Fifty strains, isolated from 44 individuals were studied in detail. None reduced methyl-

[1] This investigation was supported by a grant from the Chemical Foundation, Inc. of New York City. Read in abstract before the Thirty-sixth Annual Meeting of the Society of American Bacteriologists, Chicago, December 27, 1934.

[2] All of the numbered strains mentioned in this article came from these investigators, those in which the letter precedes the number (as in H59) from Dr. Lancefield and those in which the number comes first (as in 22E) from the Drs. Seegal.

ene blue, hydrolyzed sodium hippurate or fermented sorbitol. All but 5 fermented trehalose. Thirty-seven of the strains fermented salicin but not lactose or mannitol, thus resembling *Streptococcus equi*, according to Holman's classification (1916). Ten were like *Streptococcus pyogenes* in fermenting lactose and salicin but not mannitol, and 3 were like *Streptococcus subacidus* since they attacked none of these three carbohydrates.

The purpose of the present study was to determine the serological relationships of the strains of minute hemolytic streptococci in our possession.

METHODS

Lancefield's precipitin technique (1928a and b, 1933, 1934) was used both for grouping and typing the strains. Recently the types have been further established by the rapid slide agglutination method as described by Griffith (1934).

Precipitin tests

Antisera. Rabbits were immunized in the manner outlined by Lancefield (1933, 1934) for the production of sera high in carbohydrate antibody content. The vaccines were prepared from 18-hour broth cultures which were centrifuged at high speed and the sediments from which were resuspended in one-twentieth of the original volume of salt solution containing 0.2 per cent Formalin. They were stored in the refrigerator. After three days, subcultures were made to see whether the organisms were dead. Prior to using, the vaccines were diluted 20 times, that is, to their original volume.

Rabbits were given intravenous injections of 1 cc. of the diluted vaccines daily for six days and then allowed a week's rest. Five days after the last injection they were bled and the sera tested for precipitins. If the precipitin content was low the rabbits were subjected to another six-day course of 1 cc. injections. This cycle was repeated until the sera gave good reactions with the homologous antigens, at which time the rabbits were exsanguinated and the sera collected. Theoretically, one should use a heterologous strain of the same group in testing for group anti-

bodies but this is impossible when one is dealing with a collection of unidentified strains.

Lancefield (1934) states that, where group B strains are concerned, the sera of rabbits immunized with one or two courses of injections are usually high in group ("C" substance) antibody content but that if the injections are continued this tends to disappear or to be masked by increasing amounts of type-specific antibody. The majority of our sera were collected after two courses of injections but some gave excellent reactions after one series and were harvested then, while a few required three, or even four, courses.

Antigens. The antigens, also, were prepared according to Lancefield's directions. The streptococci from a 250 cc. broth culture were centrifuged out and resuspended in 5 cc. of 0.85 per cent salt solution. The suspension was acidified by the addition of 0.25 cc. of normal HCl, placed in boiling water for ten minutes, cooled in running water and centrifuged till clear. The supernatant fluid was neutralized to phenol red with 2 N NaOH and was kept in the refrigerator overnight. The following morning the flocculent precipitate which was usually, but not invariably, present was removed by centrifugation. The antigens were then ready for use.

Lancefield pointed out that these HCl extracts contain a number of antigenic substances and she showed that some purification could be effected by precipitation with 3 volumes of 95 per cent alcohol. In both group A and group B the group or "C" antigen remained in the supernatant fluid while the type-specific fraction precipitated out. In many of our experiments, therefore, the crude antigen was treated with 3 volumes of alcohol in the hope that a greatly purified group specific antigen would be obtained. After the addition of the alcohol the antigens were kept overnight in the refrigerator. The precipitate was removed by centrifugation and the supernatant fluid evaporated to dryness on a steam bath. The dry residue was taken up in the original volume of 0.85 per cent salt solution and, after thorough mixing, any insoluble material was centrifuged out.

Tests. Three concentrations of antigen were used in the tests,

undiluted, diluted 1:4 and 1:16. To 0.4 cc. of each dilution of antigen 0.2 cc. of serum was added, care being taken to layer it in the tube. The tests were left at room temperature for 10 to 20 minutes and examined for ring formation. They were then shaken and placed in the 37°C. water bath for 2 hours and read again. Final readings were made after they had stood in the refrigerator overnight.

Agglutinin tests

Antigens. The organisms, grown in broth for 18 to 20 hours at 37°C., were centrifuged out of the culture medium and resuspended in one-fiftieth of the original amount of broth. Most of the minute hemolytic streptococci grow diffusely in broth; those that grow granularly, however, can be got into a sufficiently smooth state for the slide agglutination test by pipetting the suspensions back and forth, particularly if the pipette is held so firmly against the bottom of the tube that considerable suction and pressure are required to force the material up and down (Eagles, 1926).

Antisera. The sera were the same as those used in the precipitin tests. Here they were used in a 1:5 dilution.

Tests. A clean slide was marked off in squares with a china marking pencil and one drop of the suspension from a capillary pipette was placed in each square. To one drop, which served as the suspension control, was added a small loopful of salt solution; to the others was added a small loopful of the 1:5 dilutions of the various sera. The serum and suspension were mixed thoroughly with the loop and, after the complete series was set up, the whole slide was rocked gently by hand. Agglutination when present was easily visible to the unaided eye. It occurred in from a few seconds to three minutes after the mixture was made.

Tryptic digestion

Several of the HCl extracts for the precipitin test were subjected to digestion with trypsin. The following method was employed:

To 5 cc. of the extract at pH 7.6 were added 100 mgm. of Fairchild's trypsin. After thorough shaking the mixture was incu-

bated at 37°C. for two hours. The activity of the trypsin was then destroyed by heating in boiling water for 10 minutes and the suspension clarified by centrifugation. Controls consisted of antigen subjected to the same procedure except that the activity of the trypsin was destroyed immediately after its addition.

RESULTS

Grouping

Preliminary tests with sera from rabbits immunized against ten strains of minute hemolytic streptococci and their corresponding antigens seemed to show (Bliss and Long, 1935) that there were at least three different groups of these organisms (table 1). One strain from each of these apparent groups was therefore chosen as the type strain and was used to immunize four rabbits. The sera from each set of four rabbits were pooled. Tests were set up with these sera against both crude HCl extracts and the alcohol-supernatant fluids of fifty-four strains of minute streptococci. The results are shown in table 2. Fourteen strains reacted predominantly with For serum, thirty-four with Hav and six with Ruf. Two of the latter, however, failed to react with Ruf serum when the alcohol-supernatant fluids were used, suggesting that the reactions obtained with the crude antigens were cross reactions and that therefore these two strains in reality belonged to a fourth group for which no serum had been prepared.

Cross reactions, as can be seen in the table, were numerous. Many of these were eliminated when purified antigens were used but with seven strains (cf. S and Gre in the table) they persisted in the tests with alcohol-supernatant fluids.

Because of the prevalence of cross reactions, which should not obtain between groups, Dr. Lancefield, whom we consulted frequently about these tests and who has been of inestimable help to us throughout the study, believed that we must be dealing with type rather than group differences,—that the main reactions were type reactions while the "cross reactions" possibly represented group relationships. She suggested that our sera might not be group-specific and, in view of this doubt, that it would be advisable to try to deprive some of the strains of their type specificity

TABLE 1

Preliminary precipitin tests with sera made from 10 strains of minute streptococci and their corresponding antigens, showing apparent existence of three groups

ANTIGENS	SERA: For	Whi	S	Holc	Hav	Ter	N	22E	W	Ruf
For	++++ ++++ ++++ D	± ++ ++	+++ D +++ D +++ D	+++ D +++ D ++++ D	+ − −	− − −	− − −	++ − −	+ + ++	+ − −
Whi	++ +++ D ++++ BD	+++ +++ ++++ D	++++ ++++ D ++++ D	+++ D ++++ D ++++ BD	± + ±	− − ST	− + +	++ ++ +	+ + +	+ + −
S	+++ +++ D ++++ BD	++++ +++ +++	++++	+++ D ++++ D +++ BD	± ++ ++	− ± −	± ++ −	++ + +	++ + +	++ + −
Holc	++++ ++++ ++++ BD	+++ +++ +++	++++ ++++ +++ D	+++ D ++++ D ++++ BD	+ − −	− − −	++ + −	++ + +	+++ + +	+++ + −
Hav	+ − −	++ ++ ++	++ D ++ D +	+ + ++ Gr	++++ D ++++ +++	++++ ++++ +++	++++ D ++++ D +++	++++ D ++++ D +++	++++ D ++++ D +++	+ − −
Ter	+ −	+ ++ −	+ + +	± ± ±	++++ D ++++ D +++	++++ ++++ +++	++++ D ++++	++++ ++++ D ++++	++++ D ++++ D ++++ D	+ − −
N	+ − −	+ ++ ++	++ ++ ++ D	+ + + Gr	++++ D ++++ D ++++	++ ++++ D ++++ D	++++ ++++ D ++++	+++ ++++ D ++++ D	++++ D ++++ D ++++ D	++ + −
W	+++ +++ +++ BD	+ ++ ++	++ ++ +	++ + ++	++++ D ++++ D ++++	++ ++++ ++++	+++ ++++ ++++	++++ ++++ ++++	++++ D ++++ D ++++ D	++++ ++ +
Ruf	+++ + −	++ ± −	++ + −	++ + ±	++ ± −	± − −	++ + −	+++ +++ +	+++ +	++++ ++ +

Twenty-hour readings—3 dilutions.
D = Disc, BD = Broken Disc, Gr = Granular, T = Turbid, ST = Slightly Turbid.

by cultivating them in immune serum and to use these altered strains for the immunization of rabbits.

This was done but the results were so confusing that they will not be described here. Suffice it to say that group-specific antisera were not acquired by this method.

In the meantime Dr. Lancefield reported to us that she had succeeded in getting an anti "C" serum with a strain, H59, which she had isolated and that nearly all of the strains which we had sent her were precipitated by it. The strains included a number each of our For and Hav "groups." She concluded that they were all members of one group and suggested that this group be designated by the letter F. At the same time she found that three other members of our Hav "group," including Hav itself and Mad and Ter were not precipitated by this serum but did come down in a serum prepared against a strain from group G, in which hitherto only large hemolytic streptococci had been placed. Hav, Mad, and Ter, then, differed in respect to "C" substance from the other strains to which they had previously seemed to be related. As will be shown when the agglutination reactions are presented this bond between Hav, etc. and the other strains lies in their having a common type specific antigen. A similar immunological relationship between heterogeneous organisms was shown by Avery, Heidelberger and Goebel (1925) to exist between pneumocococcus type II and a strain of Friedländer's bacillus.

Dr. Lancefield kindly sent us her group-specific serum and the strain, H59, with which it had been prepared and also the group G serum and group G strains. After preliminary tests with the sera, in which Dr. Lancefield's observations were confirmed, rabbits were immunized against the strains and fifty-five of our cultures were tested with the new sera. The results are shown in table 3. Forty-nine of the fifty-five strains reacted with the group F—H59 serum and ten with the group G—H13 (extracts from 4 strains precipitated in both sera). The reactions with the H59 serum ranged from ± to +++ with disc. Those with H13 were weaker, but, though the sera made with this strain were always very poor, they served to distinguish between the group F

TABLE 2

Examples of the different kinds of reactions given by crude and alcoholic extract antigens of minute streptococci in three antisera

ANTIGENS		ANTISERA: For, Ring	For, 2 hours	For, 20 hours	Hav, Ring	Hav, 2 hours	Hav, 20 hours	Ruf, Ring	Ruf, 2 hours	Ruf, 20 hours	NUMBER OF STRAINS GIVING SIMILAR REACTIONS
For	Crude	++++	++ BD	++ D	−	−	±	+	T	++	9
		++++	+++ BD	+++ D	−	−	±	±	+	+++	
		++++	+++ BD	+++ D	−	−	±	−	−	+	
	Alcohol supernatant	++	+	++ D	−	−	−	−	−	ST to +	
		+	T	++ BD	−	−	−	−	−	−	
		±	−	T	−	−	−	−	−	−	
S	Crude	+++	±	+	±	−	±	+	+++	++++	5
		+++	+++	+++ D	±	−	+	+	+	++	
		+++	+++	+++ D	±	−	++ BD	±	ST	±	
	Alcohol supernatant	±++	±	+	±	−	+	−	−	−	
		+++	++	++ D	±	−	+	−	−	−	
		+++	+++ BD	+++ D	±	−	++ BD	−	−	−	
Gre	Crude	±	−	±	++++	+	+	+	+	++	2
		±	−	±	+++	++ BD	++ BD	±	+	++	
		±	−	+	+++	++ D	+++ D	−	−	±	
	Alcohol supernatant	−	−	−	+++	−	−	−	−	ST	
		−	−	+ BD	+++	++	++ BD	−	−	−	
		−	−	+ BD	+++	++ D	+++ D	−	−	−	

Hav	Crude...............	−	−	+	++++	++	++ D	+	+	+	31
		−	−	+	++++	+++ D	+++ D	−	ST	+	
		−	−	+	++++	+++ D	++++ D	−	−	±	
	Alcohol supernatant..	−	−	−	++++	++ D	++ D	−	−	− to +	
		−	−	−	++++	+++ D	+++ D	−	−	−	
		−	−	−	+++	+++ D	++++ D	−	−	−	
Mad	Crude and alcohol supernatant........	−	−	−	++++	++	++ D	−	−	−	1
		−	−	−	++++	++ D	+++ D	−	−	−	
		−	−	−	+++	+++ BD	++++ D	−	−	−	
Ruf	Crude...............	−	−	−	−	−	−	++	T	+++	4
		−	−	+	−	−	+	++	+++	++++	
		−	−	+	−	−	±	++	++	+++	
	Alcohol supernatant..	−	−	−	−	−	−	−	T	+	
		−	−	−	−	−	−	−	−	−	
		−	−	−	−	−	−	−	−	−	
She	Crude...............	±	ST	+	−	−	T	+	T	+	2
		−	ST	+	−	−	ST	+	+	++	
		−	ST	+	−	−	−	−	−	+	
	Alcohol supernatant..	−	−	−	−	−		−	−	−	
		−	−	−	−	−		−	−	−	
		−	−	−	−	−		−	−	−	

and group G strains, since four of the six strains which did not react with H59 gave disc precipitates with H13. Two strains failed on repeated tests to react with H59 and gave only ± reactions with H13. Of these, one—H93—had been found by Dr.

TABLE 3

Precipitin reactions in anti "C" sera

ANTIGENS	SERA Group F H59	SERA Group G H13	GROUP	ANTIGENS	SERA Group F H59	SERA Group G H13	GROUP
Bay	++ D	−	F	Hall	+ D	−	F
Che	++ D	−	F	Sma	+	−	F
Gre	++ D	−	F	Ant	++ BD	−	F
Hav	++ D	−	F	D	++ BD	−	F
Hyd	++ D	−	F	For	++ BD	−	F
Jon	++ D	−	F	Holc	++ BD	−	F
Kla	++ D	−	F	Pai	−	±	F?
Merr	++ D	−	F	Rea	++ D	±	F
Mers	++ D	−	F	S	++ D	−	F
Mye	++ D	−	F	Stu	+ BD	−	F
N	±	±	F?	40E	+	−	F
Pau	++ D	−	F	84E	+	−	F
Str	++ D	−	F	Arr	+ D	−	F
Tat	++ D	−	F	Kra	+	−	F
Ver	++ D	−	F	Neu	+ D	−	F
W	++ BD	−	F	Dei	±	−	F?
MHW	++ BD	−	F	Ham	±	±	F?
Win	+ D	−	F	Moo	+ BD	−	F
Wri	++ D	−	F	Ruf	+	−	F
22E	+	−	F	She	±	−	F?
38E	+	−	F	86E	+ BD	−	F
45E	++ BD	±	F	H59	+++ D	−	F
65F	++ BD	−	F				
95E	++ D	−	F	Hav	−	+ D	G
101F	++ BD	−	F	Mad	−	+ D	G
103H	++ BD	−	F	Pen	−	+ D	G
106F	+	−	F	Ter	−	+ BD	G
Fis	++ BD	−	F	H93	−	±	G?

Lancefield to belong to group G. The other, Pai, behaves in other respects like For and presumably should be grouped with it. Four strains gave negligible or equal reactions in the two sera. One is strain N which has been placed in group F but might equally well go in group G except that it reacts more strongly with

TABLE 4

Agglutination reactions by "direct slide technique"

ANTIGENS	SERA						ANALYSIS	
	Group F				Group G			
	MHW	For	Ruf	H59	Hav	H13	Group	Type
Bay	+	–	–	–	+++	–	F	1
Cne	+++	–	–	–	+++	–	F	1
Gre	+++	–	–	–	+++	–	F	1
Hav	++	–	–	–	+	–	F	1
Hyd	+++	–	–	–	+++	–	F	1
Jon	+++	–	–	–	+++	–	F	1
Kla	+++	–	–	–	+++	–	F	1
Merr	+++	–	–	–	+++	–	F	1
Mers	++++	–	–	–	++++	–	F	1
Mye	+++	–	–	–	++	–	F	1
N	+++	–	–	–	+++	–	?	1
Pau	+++	–	–	–	+++	–	F	1
Str	++++	–	–	–	+++	–	F	1
Tat	+++	–	–	–	+++	–	F	1
Ver	+++	–	–	–	+++	–	F	1
W	+++	–	–	–	+++	–	F	1
MHW	+++	–	–	–	++++	–	F	1
Win	+++	–	–	–	++	–	F	1
Wri	+++	–	–	–	+++	–	F	1
22E	++++	–	–	–	++++	–	F	1
38E	+++	–	–	–	++++	–	F	1
45E	+++	–	–	–	++++	–	F	1
65F	+++	–	–	–	++	–	F	1
95E	+++	–	–	–	+++	–	F	1
101F	++++	–	–	–	++++	–	F	1
103H	++++	–	–	–	++++	–	F	1
106F	+++	–	–	–	+++	–	F	1
Fis	+++	–	–	–	+	–	F	1
Holl	+++	–	–	–	+++	–	F	1
Sma	+++	–	–	–	+++	–	F	1
Ant	–	+	–	–	–	–	F	2
D	–	+	–	–	–	–	F	2
For	–	++	–	–	–	–	F	2
Holc	–	++	–	–	–	–	F	2
Pai	–	+++	–	–	–	–	?	2
Rea	–	+	–	–	–	–	F	2
S	–	+	–	–	–	–	F	2
Stu	–	+	–	–	–	–	F	2
40E	–	+	–	–	–	–	F	2

TABLE 4—*Concluded*

ANTIGENS	SERA						ANALYSIS	
	Group F				Group G			
	MHW	For	Ruf	H59	Hav	H13	Group	Type
84E	–	++	–	–	–	–	F	2
Arr	–	+	–	–	–	–	F	2
Kra	–	+	–	–	–	–	F	2
Neu	–	++	–	–	–	–	F	2
Dei	–	–	+++	–	–	–	?	3
Ham	–	–	++	–	–	–	?	3
Moo	–	–	+++	–	–	–	F	3
Ruf	–	–	++	–	–	–	F	3
She	–	–	+++	–	–	–	?	3
86E	–	–	+++	–	–	–	F	3
H59	–	–	–	+++	–	–	F	4
Hav	+++	–	–	–	+++	–	G	1
Mad	+++	–	–	–	+++	–	G	1
Pen	+++	–	–	–	+++	–	G	1
Ter	+++	–	–	–	+++	–	G	1
H93	+++	–	–	–	+++	–	?	1

Ruf serum than do any of the group G strains. The other three were previously classed with Ruf.

In summary, fifty of the fifty-five strains which were studied fall into one group which is to be called group F. The remaining five belong to group G.

Typing

The readiness with which the minute streptococci produce type antibodies stood us in good stead when it came to typing them. During the process of grouping, the outlines of the type relationships were all too clearly visible. When Griffith's slide agglutination technique was applied to the strains it was seen that the agglutination and precipitin reactions paralleled each other. The results of the agglutination reactions are shown in table 4. They were absolutely clear cut.[3] Four types have so far been demon-

[3] Except for one strain, no cross reactions were seen. The excepted strain, Bul, which agglutinated in For, MHW and Hav sera was isolated just as this work

strated in group F, one like For, one like Ruf, one, represented only by strain H59 and one like MHW which comprises the largest number of strains and which is identical with the type in group G to which Hav belongs. It has been decided, in conjunction with Dr. Lancefield, to call this last type type I in both groups F and G. Type For, then is Type 2; Ruf, 3 and H59, 4.

DISCUSSION

The important point in the grouping of streptococci by Lancefield's precipitin method is the use of sera high in group or "C" antibody content. The difficulties encountered by us in the grouping of the minute hemolytic streptococci may be ascribed to two factors. First, all but one of the strains of minute streptococci were so extremely type-specific that antisera prepared against them contained no group antibody, and second, we relied upon the use of the correct antigen rather than of the correct antiserum. In doing so we exposed ourselves to two hazards. First, that the "correct antigen" might fail to react with an incorrect serum and second, that we might not recognize the "correct antigen." The latter was probably our main pitfall. Lancefield had shown that with both the group A and group B hemolytic streptococci the type and group specific antigens could be separated by precipitation with alcohol. With the group A streptococci (1928a), the type specific "M" fraction was apparently a protein and could be precipitated from the crude antigen with 3 volumes of alcohol, leaving in solution the "C" or group antigen. With the group B streptococci (1934), although the type specific fraction proved to be a carbohydrate, the same held true. The "S" or type substance again was precipitable with 2 to 3 volumes of alcohol. Therefore, at the start of the work with the minute streptococci, it was assumed that the alcoholic supernatant fluids contained the group substance in a purified state and that precipitin reactions obtained with this material were group reactions. That this was not the case was shown when a good anti "C"

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was being brought to a close and there was no opportunity to test it for agglutinin absorption. At first it was thought that perhaps the culture was a mixture of two types of minutes but strains from nine colonies behaved alike and like the parent culture. Its strongest precipitin reaction was with For serum.

TABLE 5

Precipitin reactions with four antigens and their alcoholic fractions

ANTIGENS		SERA					
		Type			Group		
Ant.	Crude	++	±	T	+	+	+
		++	+	+ BD	+	+	+
		++	++	++ D	+	−	+
	Alcohol supernatant	+	−	−	+	−	−
		+	−	+ BD	+	−	±
		−	−	++ D	−	−	+
	Alcohol precipitate	++	ST	+	+	ST	+
		++	T	+	+	±	+
		+	T	++ BD	−	−	±
D	Crude	+	−	−	+	+	+
		+	±	+	+	±	+
		+	++	++ D	+	ST	+
	Alcohol supernatant	+	−	−	+	−	ST
		+	± BD	+ D	+	−	±
		+	+	++ BD	+	−	+
	Alcohol precipitate	+	++	+ BD	+	+	+
		+	T	±	+	+	+
		0	0	0	−	T	+
Mye	Crude	0	0	0	+	T	+
		++	+	++	+	T	+
		++	+	++	±	−	+
	Alcohol supernatant	++	+	T	−	−	+
		++	+	++ D	−	−	+
		+	+	+++ D	−	−	+
	Alcohol precipitate	+	++	++	+	+	+
		+	ST	+	±	±	+
		−	−	±	−	−	±
W	Crude	+++	+++	+++ BD	+	+	+
		++	+++	+++ BD	+	±	+
		+	+++	+++ BD	−	−	±
	Alcohol supernatant	++	+ BD	+ BD	+	−	±
		++	++ BD	++ D	+	−	+ BD
		+	+ BD	+++ D	±	−	ST
	Alcohol precipitate	+++	+++ BD	+++ BD	+	−	+
		++	++	+++	+	ST	+
		+	−	+	−	−	+

serum was at last acquired and the conclusion was perforce arrived at that a part at least of the type-specific fraction of the minute streptococci resided in the alcoholic supernatant. A preliminary attempt has been made to analyse or separate the two fractions. One experiment, in which the alcoholic supernatants, precipitates and crude antigens of four strains were tested against their type and group sera, seemed to show (table 5) that

TABLE 6

Precipitin reactions of antigens before and after digestion with trypsin

Antigens		Sera	
		Type	Group
40E	Digested with trypsin	++ D	+ BD
		+++ D	+ D
		++ BD	+ BD
	Not digested	+	+
		+++ D	+ D
		+++ BD	+
Che	Digested with trypsin	+	+
		++ D	+ D
		++ D	+ D
	Not digested	++ D	+
		+++ D	+ D
		++++ D	++ D
Mye	Digested with trypsin	++ D	+ D
		++++ D	++ D
		++++ D	+ BD
	Not digested	++ BD	+
		+++ D	+ D
		++++ D	++ D

part of each antigenic substance was to be found in each alcoholic fraction. Another experiment, in which tryptic digestion was tried, indicated (table 6) that both substances might be carbohydrate since neither lost in potency by this treatment.

Another obstacle to the ready interpretation of the precipitin reactions of the minute hemolytic streptococci has been the prevalence of cross reactions. Some of these, no doubt, are attribut-

able to rudimentary group reactions in the presence of type antigen and antibody. Others cannot be explained in this way. For instance, the disc precipitates formed by the strains illustrated by S (in table 2) in Hav serum cannot be explained on this basis, since the five antigens differed in both group and type from the strain against which the serum was produced. These reactions did not occur on single occasions but were observed repeatedly. Then what is the basis for the precipitations in Ruf serum? The group G strains rarely reacted with this serum, those in group F did so constantly, and yet, if these reactions marked a group differentiation, why were they so largely eliminated by the use of

TABLE 7

SOURCE	NUMBER OF STRAINS	F				G
		1	2	3	4	1
Nephritis	16	10	6			
Rheumatic fever	3	3				
Infections caused by minute streptococci	5	4	1			
Other diseases	14	6	4	2	1	1
Normals	13	6	1	3		3
Unknown	4	13	1	1		1
Total	55	30	13	6	1	5

alcoholic extracts? The antigens of the Ruf strains behave quite differently from those of other strains. As has been said, no strain, heterologous or homologous, gave a disc precipitate with Ruf serum, though frequently the reactions were strong. In addition to this, however, only two of the Ruf strains produced discs with the anti "C" serum of strain H59. Were it not for these two strains we should be inclined to continue in our belief that the Ruf strains constitute a third group of minute streptococci. As it is (and since all the Ruf strains have the same type antigen, as shown by the agglutination reaction), they will be assigned to group F until further information is available.

Finally, there were, beside the Ruf strains, those three others

which gave negligible reactions in both the group F and group G anti "C" sera. Behaviour of this sort is explicable on the ground that such strains possess little or no group specific substance in their antigenic complex.

When the serological classification of the minute hemolytic streptococci is compared with the biological "grouping" reported in an earlier paper (Long and Bliss, 1934) no relation between the two is demonstrable, except that no members of types 1, 2 or 4 of group F belong to the second biological group, which contained the ten pyogenes-like strains.

When the source of the strains used in the present study, is compared with their serological classification (table 7) it is found that the sixteen strains from patients with glomerular nephritis, the three strains from rheumatic fever patients and the five strains which apparently were the primary cause of disease[4] all belong to type 1 or 2 of group F.[5]

SUMMARY

Fifty-five strains of minute beta hemolytic streptococci have been serologically differentiated. Fifty of the strains fall into one group which has been assigned the letter F by Lancefield. No ordinary beta hemolytic streptococci have been shown to belong to this group up to the present time. Five strains belong to group G, Lancefield. The previously isolated members of this group were ordinary beta hemolytic streptococci.

The fifty group F strains may be divided into four types. There are thirty strains in type 1, thirteen in type 2, six in type 3 and one in type 4.

The five strains of minute streptococci which fall into group G all belong to one type. The type antigen of these strains is identical with that of the members of group F, type 1.

It is difficult to group the minute streptococci because of their extreme type specificity.

[4] Paper in press by Long and Bliss.

[5] Since this paper was written a group F, type 3 strain has been recovered as the primary pathogenic agent in a case of acute pansinusitis.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

EASTERN NEW YORK BRANCH

Division of Laboratories and Research, New Scotland Avenue, Albany, April 3, 1937

An Improved Medium for the Demonstration of Hydrolysis of Sodium Hippurate by Streptococci. *Julia M. Coffey and George E. Foley*, Division of Laboratories and Research, New York State Department of Health, Albany.

A medium is described which yields uniform results in tests for hydrolysis of sodium hippurate by hemolytic streptococci. It substitutes 0.1-per-cent asparagine for 1-per-cent peptone in pepsin broth of the following composition adjusted with N/1 sodium hydroxide to pH 7.1:

Asparagine, (C.P.), Eimer & Amend	0.1%
Pepsin, (U.S.P.), Eimer & Amend	0 5%
Calcium chloride, (C.P.)	0 003%
Sodium hippurate, (C.P.), Eimer & Amend	1%
Distilled water	1000 cc.

The Relative Potency of Monovalent and Polyvalent Antimeningococcus Sera. *Mary B. Kirkbride and Sophia M. Cohen*, Division of Laboratories and Research, New York State Department of Health, Albany.

A reappraisal was made of the relative group potency and valency of monovalent antimeningococcus sera produced with recently isolated and stock meningococcus strains and of the routine six-strain therapeutic serum. Eight horses were immunized in pairs with recently isolated or stock strains of group I–III or group II. The potencies of the sera were determined by protection tests in mice and precipitation reactions with carbohydrate fractions as well as by agglutination tests with representative strains of the different groups.

As previously, no evidence was obtained of the antigenic superiority of recently isolated strains nor did the degree of virulence appear to be an index of the antigenic activity of the strains under study. The valency of the monovalent group I–III sera, within the homologous group, approximated that of the polyvalent serum; the potency, in general, fell below, especially as determined by agglutination and protection tests. In the case of the group-II sera, according to the agglutinative titers, the valency within the group appeared to be narrower; the potency approximated that of the polyvalent serum. The precipitative and protective activities were, however, definitely greater against the strains tested. Data were obtained which suggested the importance in the case of the polyvalent product of an optimum balance between groups I–III and II antigens.

ANTIGENS FOR THE COMPLEMENT-FIXATION TEST WITH ANTIGONOCOCCUS SERUM. A PRELIMINARY REPORT. *Christine E. Rice*, Division of Laboratories and Research, New York State Department of Health, Albany.

Titrations of complement-fixing activity with antigonococcus rabbit serum were made by the precise, quantitative method of Wadsworth, Maltaner, and Maltaner. Extracts of frozen and thawed gonococci or broth culture filtrates of these organisms, purified and concentrated by ultrafiltration on a 4.5 per cent nitrocellulose (Parlodion) membrane, when used as antigens, were less anticomplementary than "nucleo-protein" fractions of the same strains prepared as described by Price and Menzies, and were equally specific. Although the antigens and immune sera produced with recently isolated strains of gonococci were somewhat more specific than stock cultures of the old Torrey strains, all antigens gave marked cross reactions with antimeningococcus horse and rabbit sera and relatively weaker reactions with sera from rabbits immunized with *Neisseria flavescens* or *Micrococcus catarrhalis*. Correspondingly, all the gonococcus antisera fixed complement with extracts, filtrates, and "nucleoproteins" of meningococci.

THE EFFECT OF PHENOL AND 'MERTHIOLATE' ON THE ANTIGENIC POTENCY OF PURIFIED DIPHTHERIA TOXOIDS. *Gretchen R. Sickles*, Division of Laboratories and Research, New York State Department of Health, Albany.

Stored in the cold room with either phenol (0.4 per cent) or 'Merthiolate', 1:10,000, diphtheria toxoids purified by acetone precipitation retained their antigenic potency for six years; others purified by adsorption on calcium and alum precipitates, have been observed for two years and have retained their antigenic potency during this period. At room temperature, however, one preparation of the precipitated material stored with 'Merthiolate' had diminished in potency after two years, while another was unchanged.

The calcium and alum-precipitated preparations with 'Merthiolate', 1:10,000, as a preservative and stored at room temperature for two years inhibited growth of *Pseudomonas pyocyanea*, but when the samples were diluted to obtain a concentration of 1:20,000 of the preservative, growth was not inhibited. A similar decrease in preservative action was noted in samples of crude toxoids stored for six months at room temperature. Before storage, growth was inhibited by a concentration of 1:100,000. These precipitates appeared darkened, possibly as a result of the presence of mercuric sulphide as a decomposition product of the 'Merthiolate' compound at room temperature. The same preparation stored with 'Merthiolate' in the cold for two years, when diluted to obtain a concentration of 1:50,000 of the preservative, inhibited the growth of *Pseudomonas pyocyanea*.

DECOMPOSITION OF PNEUMOCOCCUS CARBOHYDRATE BY THE COMBINED ACTIVITY OF STRAINS OF TWO BACTERIAL SPECIES. *Myrtle Shaw*, Division of Laboratories and Research, New York State Department of Health, Albany.

Increased and broadened activity in the decomposition of pneumococcus carbohydrates was noted when two different species of microörganisms isolated from a soil sample were grown together. Both strains are aerobic. One is a Gram-negative coccus which produces smooth round colonies on

sucrose-mineral-medium agar, very slight growth on beef-extract-peptone agar, and no growth on blood agar. The other strain is pleomorphic, Gram-positive, producing extremely mucoid growth on beef extract or blood agar, but dry, irregular, yellow colonies on mineral-medium agar.

Individual colonies inoculated into mineral medium failed to decompose pneumococcus type-II carbohydrate. A mixture of both types inoculated similarly grew more heavily and utilized the carbohydrate. After further cultivation on a sucrose medium, the coccus, in pure culture, utilized the carbohydrate of type II; the other strain did not. However, the amount and rate of decomposition were increased markedly when both were grown together.

Growth of the two strains in association also broadened the activity. The coccus decomposed carbohydrates of pneumococci types II, VII, and VIII. The other strain had no action on any of the carbohydrates tested. In association, the carbohydrates of types II, III, V, VII, and VIII were utilized.

A COMPARISON OF THE EFFECT OF DIFFERENT TOXIC BACTERIAL PRODUCTS UPON THE ADRENALS OF GUINEA PIGS. PRELIMINARY REPORT. *Calvin C. Torrance*, Division of Laboratories and Research, New York State Department of Health, Albany.

The adrenals of guinea pigs which had died from intoxication with tetanus and botulinus toxins and toxic filtrates of the meningococcus were examined for their ascorbic-acid content by the method of Bessey and King. The results were compared with those obtained in a similar study of these organs from animals injected with diphtheria toxin. The vitamin-C content was found to be diminished approximately 85 per cent by diphtheria, 65 per cent by tetanus, and 50 per cent by botulinus toxins, when compared with uninjected animals of similar weight and dietary history. Toxic filtrates of the meningococcus produced an effect paralleling that of diphtheria toxin—84-per-cent reduction. Following the injection of the various bacterial products, the weight of the adrenals increased over that of the controls, diphtheria 85 per cent, tetanus 31 per cent, botulinus 13 per cent, and meningococcus 10 per cent.

From these findings it does not appear that the effect of diphtheria toxin on vitamin C is unique. Attention is drawn to the fact that the ascorbic acid was decreased more than 80 per cent in the adrenals of animals injected with the two toxic products studied which customarily produce hemorrhage; this suggests the well-known effect of generalized depletion of vitamin C following dietary deficiency.

NEW YORK CITY BRANCH

NINTH MEETING, CORNELL MEDICAL COLLEGE, NEW YORK CITY, MAY 11, 1937

THE METHOD OF ELECTRICAL CONDUCTIVITY APPLIED TO STUDIES ON BACTERIAL METABOLISM. *James B. Allison, J. A. Anderson, and William H. Cole*, Bureau of Biological Research, Rutgers University.

Data obtained through the analysis of skim milk inoculated with *Pseudomonas fluorescens* demonstrate that increase in specific conductivity of the medium is directly proportional to the amino nitrogen and ammonia formed. The rate of the ammonia formation is 42 per cent of the rate of amino nitro-

gen formation. A new lipolytic organism isolated from cream by Anderson was grown in a 1 per cent peptone medium. Analyses made on this medium show that the change in specific conductivity is also directly proportional to the formation of ammonia. There is, however a marked change in the proportionality constant near the end of the growth period. Lack of knowledge of the mechanism of coordination makes empirical relationships between other variables, such as increase in CO_2, pH, growth etc., and specific conductivity difficult to interpret.

Measurements made on skim milk inoculated with *Lactobacillus odontolyticus* demonstrate that specific conductivity increases approximately in direct proportion to a decreasing pH to the isoelectric point. The relationship between these two variables after the isoelectric point is reached depends in part upon the physical nature of the curd which is formed.

Cultivation of Throat Inclusion Bodies in Vitro. *Joan Broadhurst*, Teachers College and *Gladys Cameron*, New York University.

Human throat inclusion bodies, previously reported as occurring in the human throat, have been cultivated in chick and human tissues, heavy growths being obtained in several types of tissue cultures, including squamous epithelial cells and white corpuscles as well as fibroblasts.

The Pathogenic Staphylococcus: Its Isolation and Differentiation from Non-pathogenic Types. *George H. Chapman, Conrad Berens Lillian Curcio and Edith L. Nilson*, Clinical Research Laboratory, and laboratory of the Lighthouse Eye Clinic, New York, N. Y. Aided by grants from the Ophthalmological Foundation, Inc.

Phenol-red mannitol agar and alkaline bromthymol-blue lactose agar were inoculated with material to be tested for the presence of pathogenic staphylococci. After 10 hours incubation, yellow-zoned colonies on PRM agar were isolated and their pathogenicity confirmed by *in vitro* tests. After 48 hours incubation colonies on BTB agar were also isolated and confirmed. The results were similar in 56 per cent of the comparisons but PRM plates contained more *in vitro* positive (mannitol fermenting) colonies in 43 per cent. Both media had certain advantages.

Strains from pathologic conditions were tested for pigment production, hemolysis, coagulase, crystal-violet agar, BTB agar, mannitol and lactose fermenting and dermonecrotic properties. Typical pathogenic strains reacted positively to all. The commonest form of degeneration was loss of hemolytic power. Pigment production and coagulating power were more stable. When interpreted according to the criteria of Chapman *et al.* (J. B., **28**, 343, 1934) the results of these three tests paralleled those expected from the source and dermonecrotic properties. In some strains, regarded as intermediate in pathogenicity, these tests were negative but the other *in vitro* tests were positive.

Monilia Albicans Infection of the Human Gall Bladder and Biliary Tract with Report on Three Cases. *Frederick R. Weedon, Marie E. Shirk and Dorothy Kenney*, Bureau of Laboratories, Department of Public Health, Yonkers, N. Y.

Record of infection of the biliary tract including the gall bladder by *Monilia albicans* does not appear in

the literature, although *Monilia krusei* was reported in 1933 by Mirman. Examination of bile obtained by Rehfuss tube from 14 cases of typical gall bladder disease resulted in finding *Monilia albicans* in three cases. Since the first, or "A" bile may be contaminated by organisms from the duodenum, little importance is attached to its examination except that it in turn may contaminate the "B" and "C" bile fractions from the gall bladder and hepatic ducts which follow it. In all these cases "A" bile was obtained which did not contain yeast while the "B" and "C" bile contained organisms which were typical of *Monilia albicans*, morphologically, culturally and in respect to pathogenicity for rabbits.

LABORATORY STUDIES IN ACTINOMYCES. PRODUCTION OF THE DISEASE IN WHITE MICE WITH PUS FROM HUMAN CASES. *Frederick R. Weedon and Florence E. D. Knacke*, Bureau of Laboratories, Department of Public Health, Yonkers, N. Y.

Pus from each of five human cases of actinomycosis has been injected intraperitoneally into adult white mice and has produced a condition very similar to that seen in visceral actinomycosis in man, including the production of typical "sulphur-granules," sinus formation, cachexia and death. From the mouse lesions have been recovered morphologically typical actinomyces and with the pus from these mouse lesions the condition has been produced in a second group of mice. A third passage has also been successful.

One hundred mice were injected with pus varying in amount from 0.5 cc. to the small quantity which could be recovered from a dried cotton swab. Thirty-five mice acquired the disease, 15 died of acute peritonitis in from one to eight days and 50 were negative. In three human cases characteristic lesions in mice were produced with pus obtained before heavy treatment of the patients with iodines was begun but no such lesions were produced with pus obtained after treatment, even though actinomyces were still present in the draining fluid. These negative mice are included in the above figures.

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